Elemental sulphur metabolism in plants and defence against pathogens

Williams, Jane S.

Award date:
2002

Awarding institution:
University of Bath

Elemental Sulphur Metabolism in Plants and Defence Against Pathogens

Submitted by
Jane S. Williams

For the degree of Ph.D.
University of Bath
2002

Signature

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Contents

Acknowledgements v

Abbreviations vi

List of Figures x

List of Tables xiv

Abstract xv

1 General Introduction 1

1.1 Host-resistance, non-host resistance and tolerance 1

1.2 Recognition of the pathogen and downstream signalling 4

1.3 Expression of the plant defence responses 5

1.4 Structural defence mechanisms 5

1.5 Chemical defence mechanisms 8

1.6 The aims of the project 34

2 Quantitative Analysis of Elemental Sulphur from Biological Materials by Gas Chromatography – Mass Spectrometry 36

2.1 Introduction 36

2.2 Materials and Methods 37

2.2.1 Plant and fungal samples for analysis 37

2.2.2 Sample preparation 37

2.2.3 Internal standard and calibration curve production 38

2.2.4 GC-MS instrumentation and analysis 38

2.2.5 Calculations of S_8 content 38

2.3 Results 39

2.3.1 Thermal dissociation and recombination of $^{32}S_8$ and $^{34}S_8$ molecules 39

2.3.2 Calibration curves 41

2.3.3 Analysis of plant material 41

2.4 Discussion 44

3 Detection of Elemental Sulphur in Compatible and Incompatible Interactions Involving Fungal and Bacterial Vascular and Leaf Diseases 47

3.1 Introduction 47

3.2 Materials and Methods 50

3.2.1 Plant Growth 50

3.2.2 Inoculation of plants with fungal vascular pathogens 51

3.2.3 Inoculation of plants with bacterial vascular pathogens 52

3.2.4 Inoculation of plants with bacterial leaf pathogens 54

3.2.5 Reisolation and quantification of *V. dahliae* from the stems of tomato plants following root-inoculation 56

3.2.6 Dissection and storage of plant material in preparation for extraction of elemental sulphur 57

3.2.7 Material sent from other sources 59

3.2.8 Elemental sulphur detection by GC-MS 62

3.2.9 Localisation of sulphur by coupled SEM-EDX 62

3.3 Results 64

3.3.1 Elemental sulphur analysis in plants inoculated with fungal vascular pathogens 64

3.3.1.1 Tomato vs. *V. dahliae* 64

3.3.1.2 Cotton vs. *V. dahliae* 71

3.3.1.3 Strawberry vs. *V. dahliae* 73

3.3.1.4 Tobacco vs. *F. oxysporum* f. sp. *nicotianae* 74

3.3.1.5 French bean vs. *F. oxysporum* f. sp. *phaseoli* 77

3.3.2 Elemental sulphur analysis in plants inoculated with bacterial vascular pathogens 79

3.3.2.1 Tomato vs. *R. solanacearum* 79

3.3.2.3 Maize vs. *E. stewartii* 81

3.3.3 Elemental sulphur analysis in plants inoculated with bacterial leaf pathogens 83

3.3.3.1 Various plants vs. incompatible *P. syringae* pathovars 83

3.3.4 Elemental sulphur analysis in plants inoculated with fungal leaf pathogens 86

3.3.4.1 *B. oleracea* vs. *Peronospora parasitica* 86

3.3.5 Elemental sulphur analysis of *V. dahliae* 87

3.4 Discussion 88

4 Elucidation of the Biosynthesis of Elemental Sulphur in Tomato by Investigating Gene Expression 96

4.1 Introduction 96

4.2 Materials and Methods 109

4.2.1 Plant material 109

4.2.2 Probes for Northern analysis 110

4.2.3 PCR amplification of DNA probes 111

4.2.4 Agarose gel electrophoresis 111

4.2.5 DNA purification from agarose gels 112

4.2.6 Quantification of DNA 112

4.2.7 RNA extraction 112

4.2.8 Ethanol precipitation of RNA 113

4.2.9 Standardising the concentration of aqueous solutions of RNA 114

4.2.10 Formaldehyde gel electrophoresis of RNA 114

4.2.11 Northern Blotting 115

4.2.12 Radiolabelling of probes and Northern hybridisation 116

4.2.13 Stripping of Northern blots 117

4.2.14 Summary of techniques to clone differentially expressed genes 117

4.3 Results 119

4.3.1 Changes in the expression of genes involved in normal plant sulphur metabolism in resistant tomato plants challenged with *V. dahliae* 119

4.3.2 Genes up-regulated in resistant tomato plants in response to *V. dahliae* as determined by suppression subtractive hybridisation or cDNA-AFLP analysis 119

4.4 Discussion 123

5 Toxicity of Elemental Sulphur to Bacterial and Fungal Plant and Non Plant Pathogens 133

5.1 Introduction 133

5.2 Materials and Methods 136

5.2.1 Source, growth and maintenance of pathogens to be tested for susceptibility to elemental sulphur and the diseases that they cause 136

5.2.2 Growth of pathogens in preparation for bioassays 142

5.2.2.1 Production of spores from *Verticillium* and *Fusarium* spp. 142

5.2.2.2 Production of spores from other fungal pathogens 142

5.2.2.3 Production of bacterial cultures 144

5.2.3 Bioassays 144

5.3 Results 149

5.3.1 Effect of elemental sulphur on spore germination: Slide bioassay 149

5.3.2 Inhibition of spore germination and germ tube growth by elemental sulphur: TLC bioassay 160

5.3.3 Inhibition of mycelial growth by elemental sulphur: Disc bioassay 163

5.3.4 Toxicity of elemental sulphur to bacteria and *U. maydis* in liquid culture 165

5.4 Discussion 168

6 General Discussion 184

References 193

Appendix 1 Media 246

Appendix 2 Molecular Biology Reagents and Solutions 250

Appendix 3 Temporal effects of *V. dahliae* Infection on Sulphate and Thiol levels in Tomato Plants 254

Acknowledgements

Firstly I would like to thank my supervisor Dr Richard Cooper for giving me the opportunity to carry out this PhD and for all of his support and guidance through out my time in the lab and during my writing up. I would also like to thank Dr Malcolm Hawkesford, Dr Sharon Hall and Dr Jonathan Howarth at IACR Rothamsted for their collaboration with and advice on several aspects the project related to plant sulphur metabolism. Also acknowledged are Dr Mike Beale, Steve Croker and Paul Gaskin at IACR Long Ashton for allowing me to use their GC-MS and for their technical help with this machine preventing on me on many occasions from hitting it very hard! Thanks also to Ursula Potter at the University of Bath for her excellent assistance with the SEM-EDX and to Mike Skinner at the University of Bath for his guidance on growing healthy plants. I have worked with two fantastic undergraduate project students, Dawn Muddyman and Cathy Keeling who both contributed to some of the data in this thesis and to whom I am very grateful. I would also like to give thanks to the BBSRC for funding my research and to BSPP, SGM and the University of Bath for providing me with the funding to attend several national and international conferences.

There are several people who have definitely been detrimental to my lab work and writing up, but to whom I have to thank for fantastic times and many memories during my PhD. I would like to Sally Adams for turning me into an alcoholic and letting me sleep on her floor for three years, Ben Kemp and Paul Jones for their constant ranting and picking on me for no good reason, Paul Dean for letting me beat him at snooker (twice!!!), Catherine Bushell, Andrea Dowling and Sian Roberts for being great mates and all of the rest of lab 1.52. I would also like to thank the Salisbury lot particularly Karen S and Karen M and the Boarding House crew in Newquay for keeping me sane but not sober at weekends.

I would like to thank my parents and my nan for their constant support and essential shopping trips in Bath. Last but not least I would like to thank Darren for his endless patience, encouragement and stupid jokes without which this PhD would have been far more traumatic.

Abbreviations

°C	degree(s) Celsius
µg	microgram(s)
µL	microlitre(s)
µm	micrometre(s)
µM	micromolar
µmol	micromole(s)
400X	400 times magnification
A_{260}	absorbance at 260 nm
acetylCoA	acetyl coenzyme A
AFLP	amplified fragment length polymorphism
APS	5'-adenylylsulphate / adenosine 5'-phosphosulphate
ATP	adenosine 5'-triphosphate
***avr* gene**	avirulence gene
B broth	bacto-peptone broth
BGT agar	bacto-agar glucose triphenyl-tetrazolium chloride agar
bp	base pair(s)
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
cfu	colony forming unit(s)
cm	centimetre(s)
CSase	cysteine synthase
CzV8CS	Czapek dox V8 complete supplement
d	day(s)
dCTP	deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DMI	demethylation inhibitory
DNA	deoxyribonucleic acid
dpi	day(s) post-inoculation
ED_{50}	effective dose of a compound that reduces germination or germ tube growth of a fungus by 50%
EDTA	ethylenediamine tetra-acetic acid disodium salt
ESP	epithiospecifier proteins
EST	expressed sequence tag
f. sp.	forma specialis

g	gram(s)
g	gravity
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
Gga	*Gaeumannomyces graminis* var. *avenae*
Ggt	*Gaeumannomyces graminis* var. *tritici*
GSH	reduced glutathione
GSSG	oxidised gluthathione
GST	glutathione *S*-transferase
h	hour(s)
HPLC	high-performance liquid chromatography
HR	hypersensitive response
HRGP	hydroxyproline-rich glycoprotein
ID	inside diameter
kb	kilobase(s)
kDa	kilodalton(s)
kPa	kilopascal(s)
L	litre(s)
LRR	leucine-rich repeat
LTP	lipid transfer protein
m	metre(s)
M	molar
m/z	mass/charge
Mb	megabase(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mM	millimolar
MOPS	3-(*N*-morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
MS	mass spectrometer/mass spectrometry
ms	millisecond(s)
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced
ng	nanogram(s)
nm	nanometre(s)

nmol	nanomole(s)
NMR	nuclear magnetic resonance
NYGA	nutrient yeast glycerol agar
NYGB	nutrient yeast glycerol broth
OAS	*O*-acetylserine
OASTL	*O*-acetylserine(thiol)lyase
OD	optical density
OTC	L-2-oxothiazolidine-4-carboxylic acid
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
Pda	pisatin demethylase
PDA	potato dextrose agar
Pda^{LL}	long lag and low activity of pisatin demethylase
Pda^{SH}	short lag and high activity of pisatin demethylase
Pda^{SM}	short lag and moderate activity of pisatin demethylase
PEP^{D}	pea pathogenicity dispensible chromosome
PGIP	polygalacturonase-inhibiting protein
PLY	prune lactose yeast
PLYA	prune lactose yeast agar
PR protein	pathogenesis-related protein
psi	pounds per square inch
pv.	pathovar
***R* gene**	resistance gene
RACE	rapid amplification of cDNA ends
RIP	ribosome inactivating protein
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
s	second(s)
S^{0}	elemental sulphur
S_8	orthorhombic α elemental sulphur
SAM	*S*-adenosylmethionine
SAR	systemic acquired resistance
SAT	serine acetyltransferase

SDS	sodium dodecyl sulphate
SE	standard error of the mean
SEM	scanning electron microscopy / scanning electron microscope
SEM-EDX	scanning electron microscopy – energy dispersive X-ray microanalysis
SIM	single ion monitoring
spp.	species
TIGR	The Institute for Genomic Research
TLC	thin layer chromatography
ToF-SIMS	time of flight – secondary ion mass spectrometry
Tris	tris(hydroxymethyl)-aminomethane
UDA	*Urtica dioica* agglutinin
UV	ultraviolet
V	volt(s)
v/v	volume by volume
var.	variety
vs.	versus
W	watt(s)
w/v	weight by volume
wt	weight
XP	xylem parenchyma

List of Figures

Figure 1.1 The basic structure of glucosinolates 17

Figure 1.2 Glucosinolates, their intermediate and final degradation products 18

Figure 1.3 Accumulation of phaseollin in French bean inoculated with compatible and incompatible races of the fungus *C. lindemuthianum* 30

Figure 1.4 Elemental sulphur (S^0) 32

Figure 1.5 Accumulation of sulphur in a contact xylem parenchyma cell of a resistant, *V. dahliae*-inoculated, *T. cacao* plant 33

Figure 2.1 GC-MS mass spectra of standard sulphur solutions 40

Figure 2.2 Standard response curve for $^{32}S_8$ using $^{34}S_8$ as an internal standard 41

Figure 2.3 GC-MS chromatograms of resistant tobacco xylem challenged with *F. oxysporum* f. sp. *nicotianae* 43

Figure 3.1 The "clapper" used to inoculate maize plants with *E. stewartii* 54

Figure 3.2 Relationship between optical density (A_{600}) and colony forming units in a suspension of *P. syringae* pv. *tomato* 55

Figure 3.3 Initial symptoms expressed by susceptible (GCR 26) tomato plants inoculated with *V. dahliae* at 14 dpi 64

Figure 3.4 Colonisation of susceptible (GCR 26) and resistant (GCR 218) tomato plants inoculated with *V. dahliae* 65

Figure 3.5 Vascular occlusion of susceptible (GCR 26) and resistant (GCR 218) tomato plants inoculated with *V. dahliae* at 20 dpi 66

Figure 3.6 GC-MS analysis for S_8 of xylem tissue from resistant and susceptible tomato plants inoculated with *V. dahliae* 67

Figure 3.7 Relative sulphur levels in resistant and susceptible *V. dahliae*-inoculated and control stems of tomato plants 69

Figure 3.8 Distribution of sulphur in vascular tissues of *V. dahliae*-inoculated and control stems of tomato plants 70

Figure 3.9 Symptoms expressed by susceptible (CS50) cotton plants inoculated with *V. dahliae* at 15 dpi 72

Figure 3.10 GC-MS analysis for S_8 of xylem tissue from intermediate resistant and susceptible cotton plants inoculated with *V. dahliae* 73

Figure 3.11 Symptoms expressed by susceptible (Hapil) strawberry plants inoculated with *V. dahliae* at 21 dpi 74
Figure 3.12 Symptoms expressed by susceptible (86-4) tobacco plants inoculated with *F. oxysporum* f. sp. *nicotianae* at 35 dpi 75
Figure 3.13 Vascular browning of xylem tissue extracted from the lower stem of susceptible (86-4) tobacco plants inoculated with *F. oxysporum* f. sp. *nicotianae* at 35 dpi 76
Figure 3.14 GC-MS analysis for S_8 of xylem tissue from intermediate resistant and susceptible tobacco plants inoculated with *F. oxysporum* f. sp. *nicotianae* 77
Figure 3.15 Symptoms expressed by susceptible (Olathe) French bean plants inoculated with *F. oxysporum* f. sp. *phaseoli* at 22 dpi 78
Figure 3.16 GC-MS analysis for S_8 of xylem tissue from intermediate resistant and susceptible French bean plants inoculated with *F. oxysporum* f. sp. *phaseoli* 79
Figure 3.17 Symptoms expressed by susceptible (Super Marmande) tomato plants inoculated with *R. solanacearum* at 13 dpi 80
Figure 3.18 GC-MS analysis for S_8 of xylem tissue from intermediate resistant and susceptible tomato plants inoculated with *R. solanacearum* 81
Figure 3.19 Symptoms expressed by susceptible (Jubilee) and intermediate resistant (IFSI 90-1) maize leaves inoculated with *E. stewartii* at 14 dpi 82
Figure 3.20 Leaves responding hypersensitively to challenges with an incompatible *P. syringae* pathovar 83
Figure 3.21 GC-MS analysis for S_8 in *Arabidopsis* leaves 84
Figure 3.22 Sulphur detected by an X-ray spot analysis on a mesophyll cell of an uninoculated *Arabidopsis* leaf 85
Figure 3.23 *B. oleracea* leaves challenged with *Peronospora parasitica* 86
Figure 4.1 Sulphur assimilation in higher plants 97
Figure 4.2 Gene expression in resistant tomato plants inoculated with *V. dahliae* as shown by Northern analysis 121
Figure 4.3 Expression of clone 6-5 in resistant tomato plants challenged with *V. dahliae* as shown by Northern analysis 122

Figure 4.4 A proposed pathway for elemental sulphur production in plants 130
Figure 5.1 Effect of elemental sulphur on spore germination in isolates of the strong vascular plant pathogen *V. dahliae* 150
Figure 5.2 Effect of elemental sulphur on spore germ tube growth in isolates of the strong vascular plant pathogen *V. dahliae* 150
Figure 5.3 Effect of elemental sulphur on spore germination in isolates of the weak vascular plant pathogens *V. nubilum* and *V. tricorpus* 151
Figure 5.4 Effect of elemental sulphur on spore germ tube growth in isolates of the weak vascular plant pathogens *V. nubilum* and *V. tricorpus* 151
Figure 5.5 Effect of elemental sulphur on spore germination in isolates of the strong vascular plant pathogens *V. longisporum* and *V. albo-atrum* 152
Figure 5.6 Effect of elemental sulphur on spore germ tube growth in isolates of the strong vascular plant pathogens *V. longisporum* and *V. albo-atrum* 152
Figure 5.7 Effect of elemental sulphur on spore germination in formae speciales of the strong vascular plant pathogen *F. oxysporum* 153
Figure 5.8 Effect of elemental sulphur on spore germ tube growth in formae speciales of the strong vascular plant pathogen *F. oxysporum* 153
Figure 5.9 Effect of elemental sulphur on spore germination in isolates of *Fusarium* and *Verticillium* non-vascular plant pathogens 154
Figure 5.10 Effect of elemental sulphur on spore germ tube growth in isolates of *Fusarium* and *Verticillium* non-vascular plant pathogens 154
Figure 5.11 Effect of elemental sulphur on spore germination in isolates of non-vascular plant pathogens 155
Figure 5.12 Effect of elemental sulphur on spore germ tube growth in isolates of non-vascular plant pathogens 155
Figure 5.13 Effect of elemental sulphur on spore germination in isolates of non plant pathogenic *Verticillium* spp. 156
Figure 5.14 Effect of elemental sulphur on spore germ tube length in isolates of non plant pathogenic *Verticillium* spp. 156
Figure 5.15 Germination of *V. dahliae* (tomato isolate) spores exposed to varying concentrations of elemental sulphur 159

Figure 5.16 TLC bioassays showing toxicity of elemental sulphur to *V. dahliae* (tomato isolate), *S. nodorum, C. lindemuthianum* and *F. oxysporum* f. sp. *nicotianae* spores and mycelium 161

Figure 5.17 Disc bioassay showing toxicity of elemental sulphur to *M. fructigena* mycelial growth 165

Figure 5.18 Toxicity of elemental sulphur to *P. syringae*, *R. solanacearum* and *B. cereus* 166

List of Tables

Table 2.1 Plant and fungal material harvested, extracted and tested by GC-MS 42

Table 3.1 A summary of the incompatible plant pathogen interactions used in a survey to investigate elemental sulphur production as an induced defence response 49

Table 3.2 Reisolation of *V. dahliae* from pathogen-inoculated resistant (GCR 218) and susceptible (GCR 26) tomato plants 66

Table 4.1 Source and details of DNA used as probes for Northern analysis 110

Table 4.2 Details of primers used for amplification of DNA cloned into either pGEM-T Easy or pBluescript vectors 111

Table 5.1 Source of *Verticillium* and *Fusarium* fungal vascular pathogens of plants 136

Table 5.2 Source of fungal non-vascular pathogens of plants 137

Table 5.3 Source of bacterial pathogens of plants 138

Table 5.4 Source of fungal and bacterial non plant pathogens 138

Table 5.5 Media and growth conditions for *Verticillium* and *Fusarium* spp. 140

Table 5.6 Media and growth conditions for fungal species other than *Verticillium* and *Fusarium* 141

Table 5.7 Media and growth conditions for bacterial pathogens 141

Table 5.8 Media in which fungal spores were suspended prior to application to the elemental sulphur treated TLC plate 146

Table 5.9 Toxicity of elemental sulphur to fungi as determined by ED_{50} percentage germination and ED_{50} germ tube growth 157

Table 5.10 Inhibition of fungal growth on TLC plates impregnated with elemental sulphur 162

Table 5.11 Inhibition of fungal mycelial growth on discs impregnated with elemental sulphur 164

Abstract

The occurrence of fungicidal, elemental sulphur is well documented in certain specialised prokaryotes, but has rarely been detected in eukaryotes. Elemental sulphur was first identified in this laboratory as a novel phytoalexin in the xylem of resistant genotypes of the cocoa plant, after infection by the vascular, fungal pathogen *Verticillium dahliae*. In the current work, this phenomenon is demonstrated in tomato and cotton in response to *V. dahliae*, in tobacco and French bean in response to the vascular, fungal pathogen, *Fusarium oxysporum* and in tomato in response to the vascular, bacterial pathogen *Ralstonia solanacearum*. A novel gas chromatography – mass spectrometry method using isotope dilution analysis with ^{34}S internal standard was developed to identify unambiguously and quantify ^{32}S in biological samples. In all cases accumulation of elemental sulphur was found to resemble that of organic phytoalexins with a more rapid and intensive production in the disease resistant than in the disease susceptible lines. In contrast, no accumulations of elemental sulphur were detected in the vascular tissues of strawberry challenged with *V. dahliae* or maize leaves inoculated with the vascular, bacterial pathogen *Erwinia stewartii*. Furthermore, no elemental sulphur was detected in the leaves of many plant species in response to incompatible *Pseudomonas syringae* pathovars, suggesting that elemental sulphur production may be xylem-specific and may not be induced in all plants. High levels of elemental sulphur (between 1 and 6 μg/g fresh weight leaf tissue) were found constitutively in the leaves of *Arabidopsis* plants and this is perhaps related to the high levels of sulphur-rich compounds known to occur in *Brassica* spp..

In the interaction between *V. dahliae* and tomato, levels of elemental sulphur detected in the resistant variety (approximately 10 μg/g fresh weight excised xylem) were fungitoxic to *V. dahliae in vitro* (spore germination was inhibited >90% at approximately 3 μg/mL). Scanning electron microscopy – energy dispersive X-ray microanalysis confirmed accumulation of sulphur in vascular but not pith cells and in greater amounts and frequency in the disease-resistant genotype. More intensive localisations of sulphur were occasionally detected in xylem parenchyma cells, vessel walls, vascular gels and tyloses, structures in potential contact with and linked with defence to *V. dahliae*.

Increases in the expression of a sulphate transporter, APS reductase, cysteine synthase and glutathione synthetase in the xylem of disease-resistant lines of tomato in response to *V. dahliae* at 7 days post-inoculation may be linked to increases in the concentrations of sulphate, glutathione and cysteine also detected in these tissues after infection. In addition, a clone of unknown function was identified and shown to be up-regulated specifically in response to *V. dahliae* in resistant tomato plants. These changes in gene expression and levels of sulphate, and thiols, occurred prior to the peak in elemental sulphur accumulation, and may indicate a perturbation of sulphur metabolism induced by elemental sulphur formation. This is discussed in terms of possible elemental sulphur biogenesis.

Elemental sulphur was shown to be toxic to a wide range of fungal plant pathogens including Imperfect fungi, Ascomycetes and a Basidiomycete, falling within the magnitude generally common for phytoalexins in *in vitro* bioassays. There was no evidence for elemental sulphur tolerance as a component of pathogenicity or virulence in vascular-invading fungi, as species, isolates or formae speciales of *Verticillium* and *Fusarium* able to colonise the vascular tissues of plants were no more resistant to elemental sulphur than those that could not. Gram-positive and Gram-negative bacteria and the Oomycete *Phytophthora palmivora* were able to grow in the presence of high levels of elemental sulphur and possible mechanisms of tolerance are presented. Disparities became evident between different bioassays with this highly hydrophobic phytoalexin; these are considered in relation to the nutrient conditions in host xylem, the form of sulphur and the form of the pathogen colonising the xylem.

Chapter 1
General Introduction

1.1 Host-resistance, non-host resistance and tolerance

Plants, although static organisms, are able to defend themselves against attack and therefore most plants are resistant to most plant pathogens. Relatively few specialised pathogens have evolved mechanisms to colonise successfully a plant and cause disease. The majority of encounters between plants and potential pathogens result in basic incompatibility known as non-host resistance, where a plant species is resistant to a specific pathogen. This is in contrast to host resistance which is expressed by certain genotypes within a plant species that is otherwise susceptible or compatible (Heath, 2000a). Host resistance is usually pathogen-specific, meaning that it is restricted to a particular pathogen species and often is expressed against specific pathogen genotypes. Both forms of resistance are under genetic control that may involve one (monogenic resistance) or more genes (multigenic resistance) in the host (Heath, 2000b). The most extensively studied of all of these forms is monogenic host resistance where there is a direct or indirect interaction between the product of a plant resistance (*R*) gene and the complementary product of the corresponding pathogen avirulence (*avr*) gene (Dangl and Jones, 2001). For example the *Cf9* resistance gene in tomato mediates specific resistance only towards the races of the fungal pathogen *Cladosporium fulvum* that carry the *avr9* gene (Marmeisse, 1993). This is also commonly referred to as the gene-for-gene interaction first described by Flor (1956; 1971) and has been the principal method of breeding for disease resistance in crops for most of the 20th century (Rommens and Kishore, 2000).

Non-host resistance and multigenic host resistance have been far less extensively studied than the gene-for-gene interaction. However it appears that all these forms of resistance involve similar signalling pathways and mechanisms of resistance following initial pathogen recognition that eventually lead to localisation of the pathogen and arrest of further invasion (Somssich and Hahlbrock, 1998; Grant and Mansfield, 1999; Romeis et al., 1999; Heath, 2000b).

Tolerance has been defined as the ability of a plant to be relatively free of symptoms in spite of considerable colonisation by the pathogen (Holliday, 1989) and has often been used to describe vascular interactions among others. Due to

the focus placed on vascular diseases in this project and the widespread use of the term tolerance in the literature concerning vascular diseases its definition is addressed further here. There has been much debate as to whether tolerance involves distinct mechanisms from those which localise the pathogens in disease resistance, such as a physiological tolerance to the activity of the pathogen. Such a system was suggested by Robb et al. (2001) on discovering that a non-host isolate of *Verticillium dahliae* (E6), previously described as non-pathogenic on tomato based on a lack of symptoms, was found to colonise tomato plants both resistant and susceptible to the host isolate of *V. dahliae* (V.d.1). Quantification of fungal DNA revealed levels of E6 extended into the V.d.1-susceptible range even though symptoms were equivalent to or less than those observed in the V.d.1-resistant interactions. Tolerance has also been suggested for *Solanum chacoense* in response to *Verticillium albo-atrum,* where no visible symptoms were evident despite significant stem invasion as determined by the colony forming units (cfu) present in extracted plant stem sap (Lynch et al., 1997). In general there appears to be little hard evidence for tolerance, perhaps because statistically reliable data on the degree of colonisation in relation to the degree of symptom expression for many interactions has not been collected. In the studies by Robb et al. (2001) and Lynch et al. (1997) mentioned above, no information was given on proportion of vessels colonised, and in the study by Lynch et al. (1997) no comparison was made with a susceptible interaction making it impossible to rule out pathogen localisation in either case.

In the majority of studies where data have been collected for colonisation and symptom analysis, resistance by localisation has proved to be the mechanism. Gao et al. (1995) examined multigenic and monogenic interactions in eight cultivars of tomato inoculated with either one of three races of *Fusarium oxysporum* f. sp. *lycopersici* or *F. oxysporum* f. sp. *pisi.* There was no indication of tolerance, because extent of colonisation determined the degree of symptom expression. Similar results have also been found for tomato and potato vs. *Ralstonia solanacearum* (Grimault and Prior, 1993; Fock et al., 2000), cotton and hop vs. *V. dahliae* (Talboys, 1958; Garber and Houston, 1966; Ashworth, 1983), tomato vs. *V. albo-atrum* (Tjamos and Smith, 1975; Cooper and Wood, 1980), pea vs. *F. oxysporum* f. sp. *pisi* (Tessier et al., 1990) and many others.

In the literature the term "tolerant" seems to have been commonly misused leading to confusion. It has been used to refer to hosts that show an intermediate level of resistance to a pathogen (i.e. symptoms are reduced but not eliminated), which is actually attributable to partial localisation of the pathogen. For example Ashworth, (1983) used the term tolerant to describe cotton cultivars of Acala SJ-2 and SJ-5 to *V. dahliae*, but in the same paper stated that tolerance was due to the ability of these cultivars to inhibit the rate of development of the fungus within the plant. In some interactions with vascular pathogens colonisation analyses have shown that a small number of xylem vessels in the stem of the host can be infected without the plant showing any symptoms; for example some weed species can act as so called "symptomless carriers" of wilt diseases. In these cases the plant is referred as tolerant as it is providing a limited habitat for the growth and reproduction of that pathogen but again the pathogen is localised and not truly tolerated (Katan, 1971; Bhagwat and Duncan, 1998). In other cases the term "tolerant" has been used to describe instances where qualitative colonisation analyses such as pathogen reisolation have been used to detect systemic spread of pathogens within plants showing either mild or no symptoms (Brown and Wiles, 1970; Evans, 1971; Papadopoulos et al., 1991; Fiola and Swartz, 1994; Resende et al., 1994). However these experiments do not determine the amount of colonisation as the presence of one conidium or bacterium would give a positive result; therefore although tolerance is possible, localisation cannot be ruled out. Again many plants have been classed as symptomless carriers by these kinds of experiments. Finally there are some instances where colonisation studies have not been performed at all and plants of intermediate resistance are referred to as tolerant in comparison to more susceptible cultivars, based solely on symptom analysis (Schnathorst and Mathre, 1966; Devey and Rosielle, 1986; Gaye et al., 1991; Ray et al., 1995; Brust, 1997; Kumar, 1998). This approach has been particularly used in experiments where symptoms in many different cultivars of the same species were directly compared, such as in breeding programmes to find resistant varieties (Buddenhagen, 1981).

For these reasons the term "tolerant" has been avoided in this thesis because in none of the plant—pathogen interactions used has true tolerance been demonstrated, despite what the literature may suggest.

1.2 Recognition of the pathogen and downstream signalling

Recognition of a potential pathogen is essential for the initiation of defence responses and this is achieved by recognition of pathogen- (exogenous) or plant cell wall- (endogenous) derived signal molecules named elicitors. Elicitors differ widely in their chemical nature ranging from proteins, oligosaccharides, glycoproteins to lipids and their derivatives (Ebel and Cosio, 1994; Ebel and Mithöfer, 1998). In gene-for-gene interactions elicitors are specifically encoded by the pathogen's *avr* gene, and the host *R* gene specifies a component of the recognition event (Staskawicz et al., 1995; Dangl and Jones, 2001). In non-host resistance it is assumed that non-specific elicitors i.e. elicitors that can induce various defence responses in a large variety of plant cultivars and species, are the prime inducers of defence responses (Heath, 2000a). In most cases of host resistance and all cases of non-host resistance elicitors are believed to interact with high affinity, receptor-like binding proteins located extracellularly on the plasma membrane of host cells (Ebel and Mithöfer, 1998). However for major groups of Gram-negative plant pathogenic bacteria belonging to the genera *Erwinia*, *Pseudomonas*, *Ralstonia* and *Xanthomonas* that utilise the type III protein secretion system, a pilus-like bridge is thought to form between the bacterial and plant cells. This would provide a channel to deliver elicitors into host cells, where they interact with intracellular receptors (Bonas and Van den Ackerveken, 1997; Roine et al., 1997).

During pathogen recognition, complex signalling events are initiated in the host that activate multicomponent defence responses at local and systemic levels resulting in rapid establishment of local resistance and development of systemic acquired resistance (SAR). Changes in ion fluxes across the plasma membrane occur within min of elicitation (Hahlbrock et al., 1995) and influx of calcium ions particularly has been correlated with the activation of defence responses (Jabs et al., 1997; Xu and Heath, 1998; Grant et al., 2000). Following this, reactive oxygen species (ROS) including hydrogen peroxide and superoxide are produced in an event known as the oxidative burst (Jabs et al., 1997). These ROS, as well as possibly being directly antimicrobial (Peng and Kuć, 1992; Baker and Oikindi, 1995), are also thought to function in defence activation (Wojtaszek, 1997; Bolwell, 1999; Grant and Loake, 2000). Both of these initial transient reactions are thought at least in part to be prerequisites for the induction of a network of signals that eventually triggers defence gene activation. Exactly what other components are

involved in this network and how they fit together in order to trigger defence mechanisms both locally and systemically appear very complex and remain largely controversial. However it is thought that various other second messengers including nitric oxide (Delledonne et al., 1998), salicylic acid, jasmonates and ethylene (Dong, 1998; Reymond and Farmer, 1998; Feys and Parker, 2000; Dong, 2001) and the allosterically regulated enzymes involved in their production (*eg.* NAD(P)H oxidases and phosopholipases) are involved in this network, as are protein kinases and phosphatases (Ebel and Scheel, 1997; Yang et al., 1997; Romeis, 2001).

1.3 Expression of the plant defence responses

Upon perception of the transduced signals an integrated set of resistance mechanisms is activated and designed to protect the host tissues from colonisation by the pathogen. As well as these induced defence mechanisms there are also a number of constitutive defence mechanisms (present in healthy plants prior to infection) that the pathogen has to overcome in order to colonise the plant successfully. All of these defence mechanisms can be further classified as structural or biochemical and are described in detail below.

1.4 Structural defence mechanisms

There are many structural barriers present within the plant constitutively that may determine whether a pathogen can invade successfully. For example the anatomy of the vascular system within a plant may determine whether a vascular pathogen may grow within it. McNabb (1974) noted that elms resistant to *Ceratocystis ulmi* had small xylem vessels that were separated in widely spaced groups which was not true of susceptible elms. In other cases the plant cuticle and cell wall, especially the secondary cell wall which is often enhanced with cutin, suberin, lignin, calcium or silicon, may act as constitutive structural barriers to those organisms that cannot breach them (Ride, 1983). For example, the resistance of mature bean plants to *Rhizoctonia solani* can at least partly be explained by the resistance of its secondary cell wall to the cell wall degrading enzymes of the pathogen (Bateman et al., 1969). Reinforcement of the cell wall is often rapidly induced on pathogen attack with the deposition of newly formed callose, lignin, phenolic compounds and hydroxyproline-rich glycoproteins (HRGPs) (Bolwell, 1993; Benhamou, 1996). ROS mediated covalent cross-linking of cell wall proteins, phenolics and polysaccharides also occurs (Bolwell, 1999; Grant and

Loake, 2000). This results in the formation of plugs of material at the penetration site known as cell wall appositions. In many cases appositions or their constituents have been correlated with resistance by acting as a barrier to pathogen penetration (Ride, 1983; O'Connell et al., 1990; Benhamou et al., 1991). For example when examining the infection of soybean roots with the root rot fungus *Phytophthora sojae*, wall appositions were produced very rapidly (<4h post-inoculation) in the resistant plants and the pathogen rarely penetrated beyond the endodermis. However in the susceptible interaction the fungus was able to colonise the plant without triggering visible plant responses in cortical cells until 10h post-inoculation (Enkerli et al., 1997).

Cell wall appositions have also been shown to be important in defence against vascular pathogens and are produced in xylem parenchyma (XP) cells immediately adjacent to infected vessels (contact cells) helping to seal off the infection site so that the pathogen cannot spread. In resistant tomato plants inoculated with the fungal vascular pathogen *F. oxysporum* f. sp. *lycopersici,* 40% of contact XP cells showed intense callose deposits compared to <8% in susceptible hosts. None of the cells with apposition layers became infected but of the remaining contact XP cells, 50% in the resistant and 90% in the susceptible became thoroughly colonised (Beckman et al., 1989). Shi et al. (1991) found that the production of appositions in cotton plants infected with *F. oxysporum* f. sp. *vasinfectum* was also more rapid in the resistant plants than the susceptible as well as being more pronounced. Fewer appositions and slower production may allow escape from defence mechanisms by the pathogen and its systemic spread in susceptible plants.

Modified wall components, mainly suberin but also lipid and phenolic compounds may be secreted; onto xylem vessel walls of both invaded and adjacent vessels, pit membranes and primary walls of contact XP cells, these are associated with resistance of plants to vascular pathogens and referred to as vascular coating (Robb et al., 1991). This phenomenon is often thought to follow cell wall apposition formation chronologically (Beckman, 2000). In alfalfa plants inoculated with *V. albo-atrum* a significant inverse relationship was found between the level of coating and the frequency of pathogen penetration at pit membranes (Newcombe and Robb, 1988). Higher levels of wall coating materials and more rapid production of the coating have been detected in resistant compared to susceptible

tomato plants inoculated with *V. albo-atrum,* again suggesting possible escape of defence mechanisms by the pathogen in susceptible hosts (Robb et al., 1987; Robb et al., 1991). Furthermore, treatment of the resistant, inoculated plants with L-α-aminooxy-β-phenylpropionic acid, an inhibitor of coating secretion, resulted in the conversion to a susceptible phenotype (Robb et al., 1989). A similar phenomenon was found in resistant and susceptible chilli plants infected with the bacterial wilt pathogen *R. solanacearum.* Vascular coating was induced in resistant plants following infection but it remained completely absent in susceptible plants (Rahman et al., 1999).

A third line of induced structural defence used by plants against vascular pathogens is the production of vascular occlusions such as tyloses, gels and gums in the vessels above the infection site, that may help to stop the transpiration stream and inhibit systemic movement of the pathogen (Beckman, 1987). Vascular gels are produced by many plants and are thought to be both the result of a swelling of the end wall and pit membranes and *de novo* synthesis of primary cell wall like materials (VanderMolen et al., 1986). They are extruded by contact XP cells through pits in response to pathogens (Shi et al., 1992). This is often followed by outgrowth of contact XP cells through pits and into the vessel lumen where they balloon out into structures known as tyloses (VanderMolen et al., 1987). Many plants are able to produce tyloses in response to vascular pathogens including tomato, hops, elm, oak, banana and squash but others such as tobacco, cotton, alfalfa, carnation, radish and pea do not (MacDonald and Hindal, 1981; Beckman, 1987; Shi et al., 1992; Beckman and Roberts, 1995; Mueller and Morgham, 1996). The ability of a plant to produce tyloses may be due to the size of the pit apertures. In plants unable to produce tyloses it is thought that the pits may be too small to allow the evagination of contact XP cells into the vessel (Beckman and Roberts, 1995) but in such plants the production of gels is thought to compensate (Beckman and Talboys, 1981). To make these vascular occlusions more resistant to chemical and physical degradation by the pathogen the gels and the tylose walls often become infused and polymerised with lignifying or suberizing agents, resulting in the brown discoloration of the xylem vessels seen microscopically that is common to vascular diseases (Beckman, 2000). This is in contrast to the vascular browning seen macroscopically in infected xylem tissue of susceptible hosts that is caused by the combined or sequential effects of pectinolytic enzymes, glycosidases and phenol oxidases resulting in increased permeability of XP cells,

mobilisation of bound substrates and oxidation of phenols followed by the liberation of oxidised phenol polymers into the xylem vessels (Pegg, 1981). The number of vessels that become occluded on pathogen attack has often been correlated with resistance; for example more occluded vessels occurred in resistant tomato plants inoculated with *V. albo-atrum* and in resistant maize plants challenged with *Erwinia stewartii* than in susceptible plants. In both cases this increased number of occluded vessels correlated with lower levels of colonisation (Tjamos and Smith, 1975; Hutson and Smith, 1980; Braun, 1990). Also, xylem vessels of banana plants inoculated with *F. oxysporum* became more rapidly occluded in resistant hosts than in susceptible hosts again suggesting escape of defences by the pathogen in the susceptible host (Beckman and Talboys, 1981). It has been noted that gels produced in susceptible interactions appear weakened and seem to shear, which may be due to their insufficient development and strengthening (with lignin and suberin), excessive distension, or enzymatic degradation by the pathogen (Beckman and Talboys, 1981; Durrands and Cooper, 1988). Furthermore, delayed production of vascular occlusions in the susceptible interaction may enhance the expression of wilt symptoms in the infected plant by plugging more xylem vessels and placing a further stress on transpiration (Beckman and Talboys, 1981).

1.5 Chemical defence mechanisms

It has become increasingly clear, over the last forty years, that resistance of plants to pathogens not only depends on structural barriers but also on chemical inhibitors or antimicrobial compounds. However that is not to say that the division between chemical and structural defences is mutually exclusive, for instance, a chemical inhibitor may function as part of a structural barrier and both components may act in synergy.

Constitutive antimicrobial compounds are chemically very diverse and are found in plants from a wide range of families. There are a number of high molecular wt compounds that may be classed as constitutive inhibitors. Lectins are proteins found widely among plants of different species that can bind reversibly to specific mono- or oligosaccharides (Van Damme et al., 1998). Because of their binding specificity lectins have the capability to serve as recognition molecules not only within the plant but also between the plant and other organisms. Although their physiological functions have not been clearly defined they have been suggested to

function in, amongst other things, plant defence against invading pathogens. There are several lines of circumstantial evidence have been put forward to back up this suggestion. Lectins have been found to have a higher affinity for certain oligosaccharides than for simple sugars such as glucose and galactose, even though these oligosaccharides are often not common or totally absent in plants, for example some plant lectins bind chitin, a typical constituent of the fungal cell wall. These lectins also appear to be very stable under unfavourable conditions in a way that strongly resembles other plant defence proteins and they are often associated with the organs that require the most protection for the survival of the plant such as resting storage organs and seeds (Peumans and Van Damme, 1995). Lectins have been suggested to play a role in defence against both bacterial and fungal plant pathogens. A seed lectin from the thorn apple, *Datura stramonium* has been shown to block the movement of normally motile bacteria at the air-water interface and this was suggested to prevent invasion of the seedling roots by blocking the chemotactic movement of the bacteria towards the germinating seedling (Broekaert and Peumans, 1986). More recently a lectin from the Solanaceae, *Cyphomandra betacea* has been shown to inhibit the growth of several plant pathogenic bacteria (Nieva Moreno et al., 1997) and a lectin from mulberry leaves induced agglutination of the pathogenic bacterium *Pseudomonas syringae* pv. *mori* (Ratanapo et al., 2001). Both lectins were found to bind specific sugar residues on the bacterial cell surface (Ayouba et al., 1994). Chitin binding lectins seem likely to play a role in plant defence against fungi. Although previous reports of an antifungal chitin-binding lectin from wheat (wheat germ agglutinin) against spore germination and hyphal growth of *Trichoderma viride* was probably due to contaminating chitinases in the lectin preparation (Mirelman et al., 1975; Schlumbaum et al., 1986), other chitin-binding lectins can affect fungi. For example nettle lectin (*Urtica dioica* agglutinin (UDA)) prepared free from chitinase, inhibited hyphal growth of *Botrytis cinerea*, *Trichoderma hamatum* and *Phycomyces blakesleeanus* (Broekaert et al., 1989). The exact mechanism of how lectins such as UDA alter hyphal growth so dramatically remains as yet undetermined. It may be due to binding or crosslinking of chitin chains thereby disrupting chitin synthesis and deposition that is required for forming and strengthening the fungal hyphal tip (Van Parijs et al., 1992); alternatively growth may be altered through disruption of the delicate balance between chitin synthesis and selective hydrolysis of preformed chitin chains that is required for apical growth of fungal hyphae (Mirelman et al., 1975; Farkas, 1979). Several other chitin

binding lectins are also thought to possess antifungal activity and therefore function in plant defence including hevein, a polypeptide from the latex of the rubber tree (Van Parijs et al., 1991) and two chitin-binding polypeptides from the seeds of *Amaranthus caudatus* (Broekaert et al., 1992).

There are many other constitutive plant proteins that confer resistance to pathogens. Hydrolases such as chitinases and glucanases are found constitutively in roots, flowers, leaves, fruits and seeds of many plants (Meins et al., 1992; Graham and Sticklen, 1993; Beffa and Meins, 1996; Patil et al., 2000; Lebeda et al., 2001). They digest the chitin and β-1,3-glucans present in fungal cell walls and directly inhibit fungal growth at the hyphal tip (Schlumbaum et al., 1986; Mauch et al., 1988; Arlorio et al., 1992). Furthermore, fragments of the fungal cell wall are released from the damaged fungal cells that are themselves elicitors of further defence mechanisms (Mauch and Staehelin, 1989). Lysozymes have also been detected constitutively in various plant species and are believed to play a role in plant resistance to both Gram-positive and Gram-negative bacterial plant pathogens (Dűring, 1993). Their hydrolytic activity is directed against the bacterial cell wall component murein, causing lysis of bacterial cells (Tsugita, 1971). In most cases plant lysozymes are bifunctional also showing chitinase activity (Majeau et al., 1990; Heitz et al., 1994).

Ribosome inactivating proteins (RIPs) are RNA *N*-glycosidases that have been found constitutively in different plant tissues of many different plant species (Barbieri et al., 1993). They are considered to be involved in defence against fungal pathogens by depurinating rRNA causing deactivation of the fungal ribosomes thereby shutting down protein synthesis and killing the cells (Ferreras et al., 1995; Pu et al., 1996; Hwu et al., 2000). Fungal ribosomes are highly sensitive to RIPs but when twenty wild type species of fungi were tested for sensitivity to barley RIP only four were inhibited (Roberts and Selitrennikoff, 1986). It was then discovered that only some RIPs possess a lectin chain that can bind to fungal cell walls to form a channel and allow the *N*-glycosidase into the cells (Zhang et al., 1999). Therefore other RIPs may have a problem in becoming internalised. It seems that many RIPs require the help of chitinases and glucanases to degrade the fungal cell wall to allow their entry into the cells. These three proteins displayed synergistic effects as antifungal agents both *in vitro* and *in vivo* (Leah et al., 1991; Jach et al., 1995).

Polygalacturonase-inhibiting proteins (PGIPs) also have been detected constitutively in a wide range of plant tissues in a variety of plant species (De Lorenzo et al., 2001). They are extracellular plant proteins capable of inhibiting fungal endopolygalacturonases. Endopolygalacturonases are in part responsible for plant cell wall degradation and tissue maceration allowing some fungi to colonise plants. PGIPs as well as enhancing the resistance of the plant cell wall to enzymic attack (Lafitte et al., 1984) also prevent complete digestion of plant cell wall fragments to monomers and instead they are maintained as oligomers, which unlike monomers, are active inducers of further defence responses (De Lorenzo et al., 1994). As circumstantial evidence for a role in defence, levels of PGIPs correlate in some cases with increased resistance of the plant to fungi. For example in raspberry the level of PGIP was maximal in immature green fruits, which are more resistant than mature fruits to fungal attack (Johnston et al., 1993). Furthermore, PGIPs share striking similarities in terms of structure and specificity with *R* gene products due to their leucine-rich repeat (LRR) structural motif (De Lorenzo et al., 2001).

There are also cysteine-rich peptides known as defensins that inhibit the growth of a broad range of fungi at μM concentrations (Broekaert et al., 1995). It is thought that defensins interact with specific high affinity binding sites in the membrane of the invading fungus. This facilitates insertion of the defensins into the membrane and subsequently ion channels are formed that cause membrane permeabilisation and inhibition of fungal growth (Thevissen et al., 1999). Most plant defensins isolated to date are seed derived and may protect the seedling during the early stages of emergence as they are only released when the seed coat is perforated by the radicle of the germinating embryo (Terras et al., 1995). Defensins have also been found constitutively in vegetative tissues but are always organ specific and most abundant in the peripheral layers of that organ. For example they are found in peripheral cells of seeds (in radish), flower organs (tobacco), leaves (pea) and tubers (potato) and therefore represent a possible first-line defence against fungi in vulnerable tissues (Gu et al., 1992; Moreno et al., 1994; Terras et al., 1995). Another group of cysteine-rich peptides are the thionins that are toxic to both fungi and bacteria (Gram-positive and Gram-negative), and are found constitutively in the seed endosperm, stems and roots of several plant species (Bohlmann and Apel, 1991; Molina et al., 1993). Thionins are positively charged and may interact electrostatically and directly with the membrane phospholipids that are negatively

charged causing either pore formation or disturbance of the membrane organisation and therefore membrane permeabilisation (Florack and Stiekema, 1994). As with defensins this would lead to inhibition of bacterial or fungal growth, although thionins cause membrane permeabilisation much more rapidly and to a higher magnitude than defensins (Thevissen et al., 1996).

Lipid transfer proteins (LTPs) have also been detected constitutively in different tissues of many plant species (Selitrennikoff, 2001). They are small proteins of about 9 kDa that have the ability to transfer phospholipids between membranes (Guerbette et al., 1999). LTPs are active against the growth of a number of bacteria and fungi and so could play a role in plant defence. Although their mechanism of action is as yet unknown, it is probably not their lipid transfer ability that causes toxicity, but instead their ability to insert themselves into cell membranes to form pores and cause membrane permeabilisation (Selitrennikoff, 2001).

Some specific proteins in the groups described above are only produced constitutively within specific tissues of plants (Beerhues et al., 1990). However the accumulation of many proteins from all the above groups (hydrolases, RIPs, PGIPs, defensins, thionins and LTPs) as well as many other proteins such as peroxidases can be induced on pathogen attack both at the infection site and systemically (Molina and Garcia-Olmedo, 1993; Florack and Stiekema, 1994; Graham and Sticklen, 1994; Broekaert et al., 1995; Girbés et al., 1996; De Lorenzo et al., 2001; Lebeda et al., 2001). These proteins can accumulate to higher concentrations in those tissues where they were produced constitutively pre-infection; for example in radish plants a low concentration of a defensin was found in healthy leaves but it increased on infection with *Alternaria brassicola* (Terras et al., 1995). Alternatively they may be induced in tissues where they were not previously active, for example in healthy tobacco plants a specific defensin was only found constitutively in flower buds, but upon fungal infection it also accumulated in leaves (Gu et al., 1992; Broekaert et al., 1995). When induced by pathogens these proteins are referred to as pathogenesis-related proteins (PR proteins) and have been grouped into 11 groups based on serological and amino acid sequence analysis (Van Loon and Van Strien, 1999; Selitrennikoff, 2001).

In many cases PR proteins have been linked to resistance. For example the leaves of cucumber infected with *Colletotrichum lagenarium* showed a 600-fold increase in chitinase activity in the area of infection. Leaves away from the site of infection also had a smaller but still significant increased chitinase activity, which was correlated to increased resistance to a challenge by *C. lagenarium,* implying a role for chitinase in systemic resistance (Métraux and Boller, 1986). Tomato plants inoculated with *V. albo-atrum* showed increased chitinase and glucanase activities in their stems in comparison to healthy plants, and these increases coincided with reduced fungal colonisation (Pegg and Young, 1981; Young and Pegg, 1981; Pegg and Young, 1982). Furthermore, French bean leaves infected with an avirulent race of *P. syringae* pv. *phaseolicola* exhibited a more rapid and substantial increase in chitinase mRNA and enzyme activity than if infected with a virulent race (Vöisey and Slusarenko, 1989). Similarly PGIP and *pgip* transcripts accumulated in *Phaseolus vulgaris* in response to *Colletotrichum lindemuthianum* (Bergmann et al., 1994) and the accumulation of mRNA was far more rapid in the incompatible interaction than in the compatible interaction (Nuss et al., 1996).

The vast majority of both constitutive and induced chemical antimicrobials are of "low" molecular wt (Mansfield, 2000). Constitutive low molecular wt antimicrobials are known as phytoanticipins (VanEtten et al., 1994a) and include those compounds that are already present in healthy plants in their biologically active form and those that are present as inactive precursors and are activated in response to tissue damage or pathogen attack, usually by enzymes that are released during cell damage. Those compounds that are synthesised from remote precursors in response to pathogen attack, as a result of *de novo* synthesis of enzymes are known as phytoalexins (VanEtten et al., 1994a). Phytoanticipins and phytoalexins comprise plant secondary metabolites produced by several different biochemical pathways and are therefore chemically a very diverse group of compounds (Grayer and Harbourne, 1994). However, the distinction between phytoanticipins and phytoalexins is not always clear because a compound may be a phytoanticipin in one species and a phytoalexin in another. For example the flavone sakuranetin is produced constitutively in leaves of blackcurrant (Atkinson and Blakeman, 1982) and of the shrub *Hebe cupressoides* (Perry and Foster, 1994) but is induced in rice leaves under pathogen attack (Kodama et al., 1992). Furthermore, a compound may be a phytoanticipin in one organ of a plant and a phytoalexin in another organ of the same plant. For example momilactone A

occurs constitutively in rice husks and stems (Kato et al., 1973; Lee et al., 1999) but is induced in leaves (Cartwright et al., 1980).

Compounds that have been classed as phytoanticipins include phenolics (*eg.* catechol and protocatechuic acid from onion and arbutin from pears), isoflavones (*eg.* luteone and wighteone from lupin), thioglucosides (*eg.* glucosinolates from *Brassica* spp.), saponins (*eg.* avenacins and avenacosides from oats), cyanogenic glycosides (*eg.* dhurrin from sorghum spp.), butyrolactones (*eg.* tuliposides from tulip), long chain fatty acids (*eg.* ω-hydroxyhexadecanoic acid from pine needles) and long chain alcohols (*eg.* 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene from avocado). All of these compounds appear to have an effect on either fungal or bacterial growth or both (Crute et al., 1985; Grayer and Harborne, 1994; Mansfield, 2000; Fahey et al., 2001; Filippone et al., 2001). However, whether they actually play a role in plant defence requires additional knowledge of the localisation and concentration of the compound relative to the localisation of the invading pathogen. The distribution of phytoanticipins within plants is often tissue specific and they are often found to be concentrated in the outer layers of plant organs suggesting that they may indeed play a role in resistance. For example in unripe avocado a high concentration of the phytoanticipin 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene exists in the peel and may play a role in defence against *Colletotrichum gloeosporioides*. *C. gloeosporioides* penetrates the cuticle but the hyphae then remained dormant until the fruit began to ripen. At this point the phytoanticipin concentration declines and disease symptoms begin to develop (Prusky and Plumbley, 1992).

Despite such evidence to suggest that phytoanticipins play a role in resistance it is still only circumstantial. Molecular genetics has been used to show that a phytoanticipin plays a role in defence in the case of saponins. Saponins are glycosylated compounds that can be divided into three major groups depending on the structure of the aglycone, which may be triterpenoid, steroid, or steroidal alkaloid (Osbourn, 1996a). Saponins are found in most plant families and are toxic towards fungi by binding sterols in their plasma membranes resulting in pore formation and membrane permeabilisation (Osbourn, 1996b; Armah et al., 1999). Bacteria and Oomycetes have only low levels of sterols in their cell membranes and therefore are not susceptible (Arneson and Durbin, 1968). The most well

studied of the saponins are avenacin and avenacoside from oats and α-tomatine from tomato.

The fungus *Gaeumannomyces graminis* var. *tritici* (Ggt) is a major pathogen of wheat and barley that infects roots and subsequently causes the "take all" disease. Oats appear to be resistant to this pathogen but are susceptible to a second variety of *G. graminis*, var. *avenae* (Gga) which can also attack wheat (Turner, 1940). The saponin avenacin was found to be located in the roots of oat but not in the roots of wheat or barley (Maizel et al., 1964). Avenacin comprises four major compounds (avenacins A-1, A-2, B-1 and B-2) (Crombie et al., 1984). The most abundant and most fungitoxic avenacin, (A-1), is located in the epidermal cells of oat roots and therefore is ideally placed to be involved in defence (Crombie and Crombie, 1986; Osbourn et al., 1994). Furthermore Ggt is relatively sensitive to avenacins whereas Gga is relatively tolerant (Turner, 1953; Crombie and Crombie, 1986). Thus the presence of avenacins in oat roots appears, albeit circumstantially, to confer resistance to Ggt but not to Gga.

The ability of Gga to tolerate avenacins and particularly avenacin A-1 is associated with the production of a detoxifying enzyme that removes glucose making it much less toxic to fungal growth. This enzyme is known as avenacinase (Turner, 1961; Crombie et al., 1986) and the avenacinase gene has since been cloned and avenacinase minus mutants produced. These mutants were no longer able to infect oats as they could not breach the epidermal tissues, but they were still fully pathogenic to wheat showing that avenacinase is not required for pathogenicity of other hosts (Bowyer et al., 1995). On the plant side, the natural oat species *Avena longiglumis* lacks avenacin A-1 and is significantly more susceptible to infection by Ggt (Osbourn et al., 1994). More recently a number of saponin-deficient (*sad*) mutants have been generated in the oat species *Avena stringosa* by disrupting genes at single loci with chemical mutagenesis. All of these mutants showed increased susceptibility to both Ggt and Gga, as well as to two root invading *Fusarium* spp. (Papadopoulou et al., 1999; Trojanowska et al., 2001). It has therefore been concluded that there is a close correlation between the ability of oats to produce avenacin A-1 and its resistance to Ggt.

Tomato plants produce the saponin α-tomatine. Although it has potent antimicrobial activity and despite there being varying levels of α-tomatine within different species of the *Lycopersicon* genus (Arneson and Durbin, 1967; Courtney and Lambeth, 1977; Juvik et al., 1982; Rick et al., 1994) there is little evidence to correlate it with disease resistance. Saponin-detoxifying enzymes known as tomatinases are produced by a number of fungal pathogens of tomato and although tomatinases from different fungi do not necessarily all work in the same way, they all destroy the ability of the saponin to cause membrane pores (Osbourn, 1996b). Tomatinases of the leaf pathogen *Septoria lycopersici* and the vascular pathogen *V. albo-atrum* have a similar mechanism of action to that of avenacinase as they all remove a β,1-2-linked glucose molecule from the saponin by hydrolysis. They are also very similar at the protein and amino acid level but have clear differences in their substrate specificities allowing interaction with different host saponins (Osbourn et al., 1995). Therefore it seems that very different fungi may employ similar degradative strategies to combat different saponins in their hosts. Targeted disruption of the tomatinase gene in *S. lycopersici* produced mutants that were more sensitive than the wild type to α-tomatine, but they were still able to grow in 1 mM α-tomatine, suggesting that non-degradative methods of tolerance may also be important in this interaction (Martin-Hernandez et al., 2000).

Of potential relevance to this project are the glucosinolates. Glucosinolates are nitrogen and sulphur-containing glycosides found constitutively mainly in plants of the order Capparales, which includes the agriculturally important *Brassica* genus of which all species are able to produce them (Kjær, 1976; Rodman, 1991a). There are also a few other taxa unrelated to the Capparales that can produce glucosinolates such as certain members of the Euphorbiaceae, and the Sterculiaceae (Gill et al., 1984; Rodman, 1991a; Rodman, 1991b). So far there are over 120 known glucosinolates (Fahey et al., 2001) that all have the same basic structure (Ettlinger and Lundeen, 1956) (Fig. 1.1).

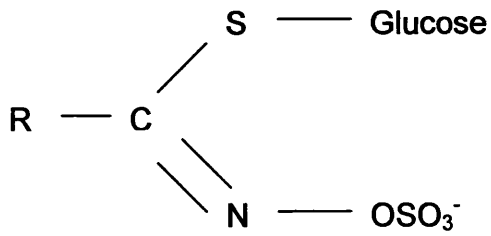

Figure 1.1 The basic structure of glucosinolates.

Their structural diversity is due largely to the R group, which may be aliphatic, aromatic or heterocyclic (indolyl) depending on the amino acids or chain elongated amino acids that they are derived from. Aliphatic glucosinolates have a side chain derived from aliphatic amino acids (often methionine but also alanine, valine, leucine or isoleucine), aromatic glucosinolates have a side chain derived from phenylalanine or tyrosine, and indolyl glucosinolates have a side chain derived from tryptophan. This diversity is further extended by secondary side chain modifications such as hydroxylations, methylations, oxidations, desaturations, sulphations and glucosylations (Halkier and Du, 1997; Chen and Andreasson, 2001; Fahey et al., 2001).

Glucosinolates are hydrolysed upon tissue damage by endogenous β-thioglucosidases known as myrosinases that always occur in plants that contain glucosinolates (Rask et al., 2000). However the glucosinolate-myrosinase system is very complex and probably has many roles within the plants. Where all of the components of the system are localised and exactly how they interact is still not clear. Myrosinases are mainly localised in specialised myrosin cells within protein-rich vacuolar like structures called myrosin grains (Thangstad et al., 1991; Hoglund et al., 1992; Xue et al., 1993). However myrosinase activity has been detected in plant tissues where myrosin cells are absent and myrosinase has been localised to cells other than myrosin cells (Bones and Inverson, 1985; Hoglund et al., 1991; Kelly et al., 1998). Also myrosinases may not always be localised in vacuoles, but certain isoforms can be localised in different subcellular compartments. Cellular localisation may be linked to the level of glycosylation, with only heavily glycosylated myrosinases being hydrophilic enough to exist in vacuoles/myrosin grains (James and Rossiter, 1991; Thangstad et al., 1993; Bones and Rossiter, 1996).

Glucosinolates may be stored with myrosinase within myrosin grains of myrosin cells along with a high concentration of ascorbic acid to inhibit glucosinolate break down (Björkman, 1976; Grob and Matile, 1979; Bones and Rossiter, 1996); alternatively the two may be stored separately, either in different compartments of the same cell, or in different cells (Bones and Rossiter, 1996; Kelly et al., 1998; Koroleva et al., 2000). The situation remains unclear and may differ between species. If the plant tissue becomes damaged, either the concentration of ascorbic acid drops activating the myrosinase, or the already active myrosinase and glucosinolate come into physical contact (Bones and Rossiter, 1996). Both allow the hydrolysis of glucosinolates by myrosinase producing glucose and an unstable intermediate. This intermediate rearranges spontaneously to produce several degradation products including isothiocyanates, thiocyanates, nitriles, elemental sulphur, epithionitriles and oxazolidine-2-thiones, dependent on the aglycone structure, pH, ferrous ion concentration and epithiospecifier proteins (ESP) (Foo et al., 2000; Chen and Andreasson, 2001) (Fig. 1.2).

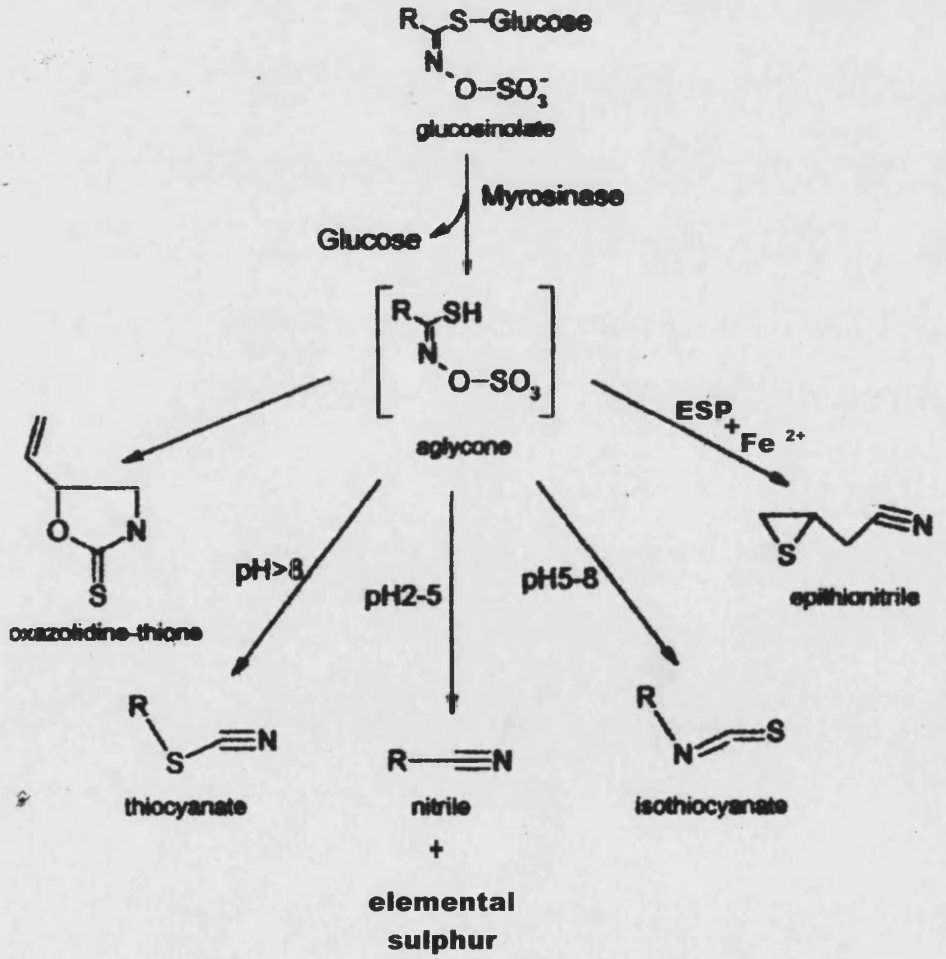

Figure 1.2 Glucosinolates, their intermediate and final degradation products. Myrosinase catalyses aglycone formation while the final products are formed non-enzymatically or via an epithiospecifier protein. Adapted from Chen and Andreasson (2001).

The main interest in glucosinolates and their breakdown products appears to be in their ability to attract or deter invertebrate and vertebrate pests feeding on *Brassica* spp. (Brown and Morra, 1997; Rask et al., 2000). Relatively few studies have been aimed at determining the significance of glucosinolates and their breakdown products in the resistance of plants to fungal and bacterial attack. They are toxic to both pathogenic and non-pathogenic fungi (Mari et al., 1993; Mayton et al., 1996; Manici et al., 1997; Hashem and Saleh, 1999; Rosa and Rodrigues, 1999) and bacteria (Brabban and Edwards, 1995; Hashem and Saleh, 1999; Rosa and Rodrigues, 1999; Lin et al., 2000). Susceptible fungal species include many *Brassica* pathogens such as *Alternaria* spp. (Milford et al., 1989), *Leptosphaeria maculans* (Mithen et al., 1986), *Peronospora parasitica* (Greenhalgh and Mitchell, 1976) and *Mycosphaerella brassicae* (Hartill and Sutton, 1980). The concentration of glucosinolates occurring in leaf tissue of *Brassica* crop plants and/or wild species was estimated to be sufficient to inhibit fungal growth *in vitro* in each case (Greenhalgh and Mitchell, 1976; Harthill and Sutton, 1980; Mithen et al., 1986; Milford et al., 1989) and so it is generally assumed that these metabolites are important in plant defence. However apart from these *in vitro* experiments there is little other evidence for their role in plant defence. Numerous attempts to correlate levels of glucosinolates to resistance to specific pathogens have failed. For example when thirty-three *Brassica napus* lines with variable leaf glucosinolate contents were assessed for susceptibility to infection by *L. maculans* and *Alternaria* spp. there was no positive relationship between the glucosinolate content and resistance. In fact for *Alternaria* spp. the opposite was true, increased glucosinolate levels coincided with increased susceptibility to the pathogen (Giamoustaris and Mithen, 1997). Furthermore, in the interaction between the club root disease causing pathogen *Plasmodiophora brassicae* and *Brassica rapa*, development of symptoms again appeared to be correlated with glucosinolate content (Ludwig-Müller et al., 1999) suggesting that these pathogens have become adapted to their host plants. Recently a gene has been characterised from *L. maculans* encoding a putative cyanide hydratase that degrades nitriles to the less toxic formamide further suggesting that adaptation of pathogens to their glucosinolate containing hosts may occur (Sexton and Howlett, 1998). It may be that glucosinolates and their breakdown products perform a role in non-host resistance where only fungi or bacteria that are able to circumvent them are able to invade the plant successfully. This may be by limiting the extent of tissue damage thus limiting release of toxic breakdown products, or by developing a

tolerance or an ability to sequester or metabolise the glucosinolates or breakdown products.

The concept that low molecular wt compounds are induced in plants in response to pathogen attack was first introduced many years ago. As long ago as 1911 Noel Bernard discovered that orchid tubers became resistant to further fungal attack after they had been infected by the fungus *Rhizoctonia repens* and that the fungus-infected tissue produced a diffusible inhibitor of fungal growth (Stoessl and Arditti, 1984). The expression "phytoalexin" was first used by Müller and Börger in the 1940s to describe a hypothetical compound produced by potato tubers in response to incompatible races of the late blight fungus *Phytophthora infestans* that induced resistance to subsequent challenges by compatible races of *P. infestans* or tuber-infecting *Fusarium* spp. (Müller and Börger, 1940). Müller (1958) followed this up by showing that French bean pod tissues also produced strongly fungistatic substances when challenged with incompatible pathogens. The first phytoalexin to be isolated and structurally characterised was pisatin from pea (Cruickshank and Perrin, 1961) and subsequently many more have been isolated and chemically characterised. There are now over 350 characterised phytoalexins from approximately 30 plant families including both monocots and dicots (Kuć, 1995).

Like phytoanticipins, phytoalexins are chemically very diverse and include indoles (*eg.* camalexin from *Arabidopsis*), isoflavonoids (*eg.* phaseollin from bean), sesquiterpenes (*eg.* rishitin from potato), stilbenes (*eg.* viniferins from grapevine), triterpenes (*eg.* arjunolic acid from cocoa), furanoacetylenes (*eg.* wyerone and derivatives from broad bean) and diterpenes (*eg.* momilactone A from rice). Like phytoanticipins, phytoalexins are toxic to bacteria or fungi or both (Grayer and Harbourne, 1994; Mansfield, 2000). Most phytoalexins are produced from remote precursors from one or more of three biosynthetic pathways: the acetate-malonate, the acetate-mevalonate and the shikimic acid pathways (the pathways responsible for synthesis of most of the housekeeping compounds vital for all plants) (Kuć, 1995; Hammerschmidt, 1999), although there are some exceptions such as elemental sulphur (Cooper et al., 1996). Individual species may produce more than one type of phytoalexin. The majority produce between two and five although the potato *Solanum tuberosum* can produce more than twenty (Bailey, 1987). Plant families often produce chemically similar types of phytoalexins, for

example the Leguminosae tend to produce isoflavonoids and the Solanaceae tend to produce sesquiterpenoids; although this is not always the case, as in the Poaceae stilbenes, deoxyanthocyanins, avenathramides and diterpenes have been reported as phytoalexins (Grayer and Harbourne, 1994; Mansfield, 2000). Within a plant family a level of specificity can also be noted, for example the sesquiterpenoid rishitin is found in tobacco and potato but not pepper, whereas the sesquiterpenoid capsidiol is found in tobacco and pepper but not potato (Kuć, 1995). Phytoalexins are produced in many plant tissues including stems, roots, leaves and fruits, although individual tissues may produce different phytoalexins (Kuć, 1995).

To implicate phytoalexins in resistance they must be present in the right place, in sufficient quantities and at the right time to inhibit the pathogen (Osbourn, 1999). It is often difficult to achieve all of these criteria. Timing, quantification and cellular localisation studies have provided evidence that supports a role for phytoalexins in the resistance of cotton to *Xanthomonas campestris* (Essenberg et al., 1992; Pierce et al., 1996) and *V. dahliae* (Mace et al., 1989), oats to *Puccinia coronata* (Mayama and Tani, 1982; Mayama et al,. 1982), soybean to *Phytophthora megasperma* (Yoshikawa et al., 1978; Hahn et al., 1985) and carnation to *F. oxysporum* f. sp. *dianthi* (Niemann et al., 1990). For example, infection of sorghum seedlings with the maize pathogen *Colletotrichum sublineolum* resulted in the accumulation of the red/orange coloured 3-deoxyanthocyanidin phytoalexins, luteolindin and apiggeninidin that were localised by light microscopy in inclusion bodies that form in the cytoplasm of the cell being infected (Nicholson et al., 1987; Snyder and Nicholson, 1990). Colourless inclusions at first appear in epidermal cells at the time of fungal appressorial maturation and then migrated towards the infection peg where they accumulate the phytoalexins and therefore take up the red/orange colour. The inclusions eventually burst and the phytoalexins are released into the cytoplasm of the infected cell in direct contact with the pathogen. The amount of phytoalexin present in the infected host cells was determined by microspectroscopy and reported to be 0.48 to 1.20 ng luteolindin and 0.24 to 0.91 ng apigeninidin per cell which was well above that required for *in vitro* toxicity to *C. sublineolum* (Snyder et al., 1991).

Few phytoalexins are coloured and so localisation of phytoalexins often proves difficult. There are other methods that have been designed to localise particular phytoalexins. Some phytoalexins are fluorescent such as wyerone derivatives in *Vicia faba* and lacinilenes in cotton and so can be localised by fluorescence emission or UV absorption (Mansfield et al., 1974; Mayama and Tani, 1982; Essenberg et al., 1992; Pierce et al., 1996). In some cases such as with flavanol, flavonoid and terpenoid aldehyde phytoalexins in cotton, histochemistry has been used for localisation (Mace et al., 1978; Mace et al., 1989; Dai et al., 1996). Laser microprobe mass analysis has been used to localise glyceollin accumulation in soybean (Moesta et al., 1982), as has pyrolysis mass spectrometry (Niemann et al., 1990). However, although localisation, timing and quantification studies provide powerful evidence that phytoalexins play a role in resistance, the evidence is still circumstantial. No studies as yet have shown that a phytoalexin is responsible for stopping a pathogen. To provide better evidence that a phytoalexin plays a role in defence, a combination of experimental approaches including molecular and classical genetic studies is required.

One critical approach to investigate the role of a phytoalexin is to analyse pathogenic isolates that are insensitive to their host phytoalexins. Many plant pathogens have been shown to detoxify, degrade or tolerate phytoalexins in their hosts (VanEtten et al., 2001). *Botrytis fabae* is a necrotrophic pathogen of broad bean leaves that causes chocolate spot disease. It invades by penetrating and degrading host epidermal cell walls leading to the death of affected cells. Areas of dead cells combine forming dark brown lesions that rapidly spread away from the infection site (Mansfield and Hutson, 1980). Other *Botrytis* spp. that are non pathogens of bean such as *B. cinerea*, *B. tulipae, B. elliptica* and *B. squamosa* are also able to penetrate epidermal cells but are far less destructive and produce only short infection hyphae that arrest within the cell walls of epidermal cells by 12h post-inoculation. Although lesions develop at the site of infection by non-pathogens they do not spread (Mansfield and Hutson, 1980). The restriction of hyphal growth of *B. cinerea* in bean leaves has been correlated with the rapid accumulation of the phytoalexins wyerone and wyeronic acid to fungitoxic levels (Mansfield, 1982). Furthermore, in the lesions produced in the susceptible interaction with *B. fabae* an initial increase in phytoalexin concentrations was followed by a subsequent decrease as the tissue became colonised with the pathogen and turned necrotic. Only a low concentration, if any, of the phytoalexins

was present in the completely necrotic lesion (Hargreaves et al., 1977; Mansfield, 1982). This suggested that phytoalexin accumulation was involved in restricting the growth of *B. cinerea* in bean leaves and that *B. fabae* was able to detoxify the phytoalexins to prevent them accumulating to fungitoxic concentrations. *B. fabae* was more tolerant of wyerone and derivatives than *B. cinerea* although both pathogens appeared to have the ability to detoxify them. Detoxification was found to be a consequence of fungal growth and so the more tolerant *B. fabae* was able to grow in the high concentrations of phytoalexins within lesions and detoxify them whereas *B. cinerea* was inhibited (Rossall and Mansfield, 1978). This suggests that non-degradative mechanisms are also involved in the tolerance of *B. fabae* to wyerone and its derivatives and it seems that this tolerance tips the balance in favour of the pathogen. The mechanisms of tolerance to wyerone and derivatives are not as yet understood but may be related to less sensitive sites present in the *B. fabae* fungal cell membrane (Rossall and Mansfield, 1978). Subsequently analysis of *B. fabae* mutants revealed that cell killing during the early stages of infection is probably more important in pathogenicity than tolerance to wyerone; rapid cell killing suppressed phytoalexin production (Hutson and Mansfield, 1980). In either case it would seem that phytoalexins play a role in defence for such mechanisms to exist.

The most detailed work on phytoalexin detoxification was conducted on isolates of the pea pathogen *Nectria haematococca.* Isolates of *N. haematococca* that were able to tolerate and demethylate the pea phytoalexin pisatin (Pda^+) were highly virulent on pea. All of the isolates that lacked this pisatin demethylation ability (Pda^-) were sensitive to pisatin and were non pathogenic (VanEtten et al., 1980). This correlation was further confirmed by analysis of progeny following sexual crossing of different isolates of *N. haematococca* that differed in their pathogenicity to pea. In all cases pathogenicity segregated with pisatin tolerance (Tegtmeier and VanEtten, 1982). Pisatin detoxification was found to be an enzymic reaction performed by pisatin demethylase (Pda). Pda has been further characterised as a microsomal NADPH-dependent cytochrome P450 (Matthews and VanEtten, 1983; Desjardins and VanEtten, 1986).

A more detailed genetic analysis has revealed that *N. haematococca* possesses multiple genes that confer characteristic ability to cause pisatin demethylation. So far seven cytochrome P450s that encode Pda have been identified in *N.*

haematococca (*pda 1, 2, 3, 4, 5, 6-1* and *6-2*) but no field isolate has been found with more than two (Miao et al., 1991a; Maloney and VanEtten, 1994; Reimmann and VanEtten, 1994; Delserone et al., 1999). Phenotypes conferred by these genes have been classified into three different classes based on the lag period for induction of Pda and the resulting amount of activity induced: Pda^{SH} = short lag and high activity, Pda^{SM} = short lag moderate activity and Pda^{LL} = long lag low activity (VanEtten and Matthews, 1984; Mackintosh et al., 1989). *Pda 1* is thought to be required for the Pda^{SH} phenotype, *pda 4* and *5* genes are thought to be required for the Pda^{SM} phenotype and *pda 2, 3, 6-1* and *6-2* required for the Pda^{LL} phenotype. Pda^{SH} and Pda^{SM} isolates are virulent on pea while Pda^{LL} isolates are no more virulent than Pda^{-} isolates that lack all *pda* genes (Kistler and VanEtten, 1984; Mackintosh et al., 1989; VanEtten et al., 1989; Miao and VanEtten, 1992; George et al., 1998). Furthermore the Pda from Pda^{SH} isolates of *N. haematococca* is selectively induced by pisatin, has a high substrate specificity for the compound and a low K_m when pisatin is the substrate (VanEtten et al., 1989; George et al., 1998). It is therefore suggested that Pda^{SH} and Pda^{SM} isolates of *N. haematococca* have evolved a specialised, readily inducible, detoxification system involving the pda^{SH} or pda^{SM} genes respectively that allows them to be more tolerant to pisatin and therefore more virulent on pea than isolates that more slowly detoxify pisatin (Pda^{LL}) or lack Pda (Delserone et al., 1999). This also suggests that pisatin plays a role in defence against isolates lacking this specialised system.

The first *pda* gene to be cloned was named *pda T9* and thought to be a homologue of *pda 1* (Weltring et al., 1988; Maloney and VanEtten, 1994; George et al., 1998). This has allowed the role of pisatin demethylation in pathogenicity to be investigated further. When this gene was transformed into a Pda^{-} isolate of *N. haematococca* three transformants were more tolerant of pisatin and two were significantly more pathogenic to pea (Ciufetti et al., 1988). Furthermore, when the gene was transformed into the maize pathogen *Cochliobolus heterostrophus* or the chickpea pathogen *Ascochyta rubiei*, they acquired the ability to rapidly demethylate and detoxify pisatin and had an increased ability to cause necrotic lesions on pea (Schäfer et al., 1989; Barz and Welle, 1992; Oeser and Yoder, 1994; Weltring et al., 1995). Thus the possession of Pda appeared to enhance pathogenicity of the fungi and furthermore pisatin seemed to play a role in restricting the growth of the wild type fungi in pea.

When the *pda 1* gene was knocked out in Pda^{SH} isolates of *N. haematococca,* despite the fact that transformants had lost their ability to demethylate pisatin, pathogenicity was only reduced and not eliminated, conflicting with results of earlier genetic studies where pathogenicity was always linked with pisatin detoxification (Tegtmeier and VanEtten, 1982; VanEtten et al., 1994a; Wasmann and VanEtten, 1996). During the course of these earlier genetic studies to characterise the *pda* genes it was found that some were located on dispensable chromosomes or dispensable regions of chromosomes (Miao et al., 1991a; Miao et al., 1991b; Miao and VanEtten, 1992). These dispensable chromosomes as well as containing pda^{SH} and pda^{SM} genes probably also contain other genes required for pathogenicity named PEP^{D} (pea pathogenicity dispensable chromosome) genes (VanEtten et al., 1994b). Naturally occurring Pda^{-} isolates and those resulting from crosses are thought to have lost this dispensable chromosome or dispensable region and therefore lost pathogenicity not only because of the loss of pda^{SH} and pda^{SM} genes but also because of the loss of other pep^{D} genes (Wasmann and VanEtten, 1996) thus explaining why Pda^{SH} or Pda^{SM} phenotypes were always previously linked to pathogenicity in crosses (VanEtten et al., 1994b). The Pda^{-} transformants are consistent with this model. *Pda 1* is located on a 1.6 Mb chromosome (Miao et al., 1991a; Miao et al., 1991b) and those transformants that have lost the whole chromosome are less virulent and more similar to Pda^{-} natural isolates than those that have only lost the *pda 1* gene (Wasmann and VanEtten, 1996). This questions the importance of pisatin demethylase in pathogenicity and also the role of pisatin in defence. It may be that pisatin makes a small contribution to defence along with many other factors, thereby causing the decreased virulence seen in *N. haematococca* Pda^{-} isolates and the increased virulence shown by non-pathogens transformed with pisatin demethylase.

It has been shown that even Pda^{-} isolates, whether naturally occurring or mutants of *N. haematococca,* are more tolerant to pisatin than other very closely related fungi. Furthermore, Pda^{-} isolates of *N. haematococca* also possess another non-degradative form of tolerance to pisatin, the mechanism of which as yet remains unknown, but may be due to a change in membrane structure or function (Denny and VanEtten, 1983; Denny et al., 1987). It may be this redundancy of tolerance mechanisms that allows Pda^{-} mutants to retain a certain degree of pathogenicity on pea despite lacking pisatin demethylase and again implicates pisatin as a defence response that has to be overcome for pathogenicity to occur.

The virulence of *Gibberella pulicaris* that causes a dry rot on potato appears to be associated with its ability to detoxify the potato phytoalexin rishitin (Desjardins and Gardner, 1991; Gardner et al., 1994). Sexual crosses between pathogenic and non-pathogenic isolates showed that pathogenicity correlated with phytoalexin detoxification and at least two loci were involved (Desjardins and Gardner, 1991). Furthermore, virulence was positively correlated with detoxification rates of rishitin *in vitro* (Weltring et al., 1998). Further studies on this interaction should eventually lead to the cloning of the fungal genes involved in rishitin detoxification and will allow the role of this phytoalexin in plants to be investigated further.

An alternative and powerful approach to determine if a phytoalexin plays a role in plant defence is to knock it out and see if defence is compromised. The only plant in which phytoalexin-deficient mutants have been obtained is *Arabidopsis* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1996; Glazebrook et al., 1997). *Arabidopsis* only produces one indole phytoalexin, camalexin, that inhibits the growth of both fungi and bacteria *in vitro* (Browne et al., 1991; Tsuji et al., 1992; Rogers et al., 1996). It accumulates to high levels in response to both virulent and avirulent *P. syringae* spp. and it fluoresces under UV so its accumulation is easy to detect. Five phytoalexin-deficient (*pad*) mutants have been found (*pad1-1, pad2-1, pad3-1, pad4-1* and *pad5-1*) that range in their ability to produce camalexin from **none** to 30% of wild type, depending on the mutant and the microorganism used to **chall**enge the plant (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). It has since been shown that some of the *pad* mutants interrupt regulation of defence responses rather than just biosynthesis of camalexin. The pleitropic nature of *pad1-1* mutants is evident by its effect on leaf morphology (Glazebrook et al., 1997) and that of *pad4-1* mutants is evident by a block in the production of salicylic acid (Zhou et al., 1998). Only the *pad3-1* mutant, which is mutated in a gene encoding a cytochrome P450 enzyme resembling an enzyme from maize involved in the production of an indole derived phytoalexin, is thought to be a true camalexin biosynthesis mutant (Zhou et al., 1999).

Analysis of disease phenotypes of various single *pad* and double *pad* mutations have shown a complex relationship between phytoalexin production and disease resistance that depends on the pathogen involved. The *pad3-1* mutant showed increased susceptibility to the fungal pathogen *A. brassicola* but not to *B. cinerea*, *P. parasitica* or *Erysiphe orontii* (Glazebrook et al., 1997; Thomma et al., 1999).

However despite a complete lack of camalexin production *pad3-1* showed no increased susceptibility to both virulent and avirulent strains of *P. syringae* (Glazebrook and Ausubel, 1994). *Pad1-1*, *pad2-1*, *pad4-1* mutants did show increased susceptibility to disease causing strains of *P. syringae*, possibly due to pleiotropic effects but even these mutants did not show increased susceptibility to strains that are usually unable to infect *Arabidopsis* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). This suggests that camalexin does not play a major role in resistance but may play a role in reducing disease symptoms particularly in response to *A. brassicola.* The concentration of camalexin required to inhibit fungal growth (20-50 μg/mL threshold concentration above which the viability drops by several orders of magnitude) was much lower than required to inhibit bacterial growth (250-500 μg/mL) (Rogers et al., 1996) and it is therefore possible that camalexin plays more of a role in resistance to fungi than bacteria. Previous studies of many phytoalexins have shown that relatively high concentrations of approximately 10^{-4} – 10^{-5}M are usually necessary for *in vitro* inhibition of pathogens (Smith, 1982). The amount of camalexin required to inhibit fungal growth falls within these concentrations, but the concentration of camalexin required to inhibit bacterial growth falls below this concentration at 10^{-3}M (Rogers et al., 1996). This questions the validity of using *Arabidopsis* and *P. syringae* to investigate the role of camalexin.

Phytoalexin accumulation has long been associated with a phenomenon known as the hypersensitive response (HR). The HR was first described by Stakman (1915) on noticing an extreme type of resistance when non-host plants were inoculated with isolates of *Puccinia graminis.* He coined the term hypersensitiveness to indicate the abnormally rapid death of host plant cells when attacked by rust fungi. It is now known that the HR is a common but not obligatory feature of the resistance response to bacteria and fungi in both gene-for-gene interactions and non-host resistance (Heath, 2000a). It has more recently been defined as "the rapid death of plant cells in association with the restriction of pathogen growth" (Goodman and Novacky, 1994). Damage to the plant is typically limited to either one or few necrotic cells at the infection site but can involve many more (Heath, 2000c).

The ubiquity of the HR, its requirement for active plant cell metabolism (Aist and Bushnell, 1991; Zeyen et al., 1995; Heath et al., 1997; Mansfield et al., 1997) and a few shared features with mammalian programmed cell death (apoptosis) (Gilchrist, 1998; Heath, 1998a) has led to the conclusion that the HR is endogenously programmed. As explained previously, the oxidative burst is an early defence response produced after pathogen recognition. Oxidative stress in plants caused by ROS can lead to severe damage to critical cellular macro-molecules including nucleic acids, proteins and lipids (Imlay and Linn, 1988; Halliwell and Gutteridge, 1989) as well as plant cell cycle arrest (Reichheld et al., 1999). ROS have also been implicated in animal cell apoptosis, which in turn has been shown to be linked to the cell cycle (Gilchrist, 1998; Green and Reed, 1998). Therefore it is widely accepted that ROS produced by the oxidative burst are involved in triggering and/or executing the HR. However some studies dispute this role. The oxidative burst caused by bacteria occurs in two phases of which phase two appears to be required for a HR (Baker and Orlandi, 1995), but this phase is also triggered by mutant bacteria that do not induce a HR in tobacco leaves or death in cell suspensions (Glazener et al., 1996). Furthermore cytological studies of cowpea challenged with the rust fungus *Uromyces vignae* could not detect any ROS generation prior to the onset of hypersensitive cell death (Heath, 1998b). The variable role of ROS in the HR is also demonstrated by the fact that ROS scavengers can inhibit cell death in some situations, for example in soybean cells inoculated with an avirulent strain of *P. syringae* pv. *glycinea* (Lamb and Dixon, 1997) but not in others (Yano et al., 1999), and so the role of ROS in inducing hypersensitive cell death may differ in different plant-pathogen interactions (Heath, 2000c). However ROS once formed are likely to play a role in the signalling of further defence responses (Bolwell, 1999; Grant and Loake, 2000) and may play a direct role in inhibition of the pathogen within the hypersensitive cells (Peng and Kuć, 1992; Baker and Oikindi, 1995).

For non-biotrophic pathogens that do not require their host cells to stay alive, cell death alone cannot be the explanation for restriction of pathogen growth. This role must be carried out by the many other induced defence responses including the modification of plant cell walls, the accumulation of PR proteins and phytoalexins (Kombrink and Somssich, 1995), and the accumulation of oxidised phenolic compounds evident by the eventual autofluorescence and browning of the dead cell(s) (Nicholson and Hammerschmidt, 1992). The induction of these defence

responses not only occurs within the dying cells but also in their adjacent living neighbours. In addition the HR typically induces systemic resistance to previously compatible pathogens throughout the plant (Kombrink and Somssich, 1995). Therefore the HR encompasses both cell death and defence gene expression (Heath, 2000b; Lam, 2001). In fact disease resistance and all of the inducible defence responses currently associated with the HR can occur without cell death and furthermore pathogens may also trigger cell death and trigger defence responses while successfully growing in susceptible tissue (Heath, 2000c).

The interaction between *C. lindemuthianum*, the anthracnose fungus, and *P. vulgaris* has provided a good model to study the role of phytoalexins in the HR. On initial infection of susceptible hosts *C. lindemuthianum* behaves as a biotroph. Epidermal cells are penetrated and large primary hyphae are produced that grow between the cell wall and plasma membrane. Colonised cells at the edge of the primary mycelium are intact but earlier infected cells eventually senesce and die. Despite the death of many cells during this stage host responses are not induced. At temperatures less than 17°C the fungus continues to grow in this state for several d extensively colonising cells, but eventually it begins to produce narrow secondary hyphae and growth switches to necrotrophy. In this phase the pathogen resorts to cell killing often well in advance of the hyphae, extensive cell wall degradation and the subsequent formation of a black lesion on the host (Bailey et al., 1980; Bailey et al., 1992). Host resistance is encoded by a gene-for-gene interaction that results in a HR at the infection site. The challenged cells plus a few adjacent cells rapidly become necrotic producing a flecked appearance on the challenged leaf and the pathogen is inhibited from further spread (Bailey and Deverall, 1971). French bean plants produce several isoflavonoid phytoalexins in response to this pathogen but the main one is phaseollin. It accumulates to the highest concentrations and is fungicidal at 3 μg/mL to germ tubes, at 10 μg/mL to germination of spores and at 22.5 μg/mL to mycelium (Bailey and Deverall, 1971). In the resistant interaction phaseollin rapidly accumulated following the death of infected cells with the concentrations within hypersensitive cells estimated to be between 3 and 4 mg/g fresh wt. This was consistent with the observed inhibition of fungal growth. In the susceptible interaction no phytoalexins accumulated during the biotrophic growth stage but they did accumulate in the necrotrophic growth phase to levels that were eventually higher than those produced by the resistant interaction (Fig. 1.3). Although these high levels occurred in the susceptible

interaction, at this stage lesions had already become established and the fungus had moved on (Bailey and Deverall, 1971).

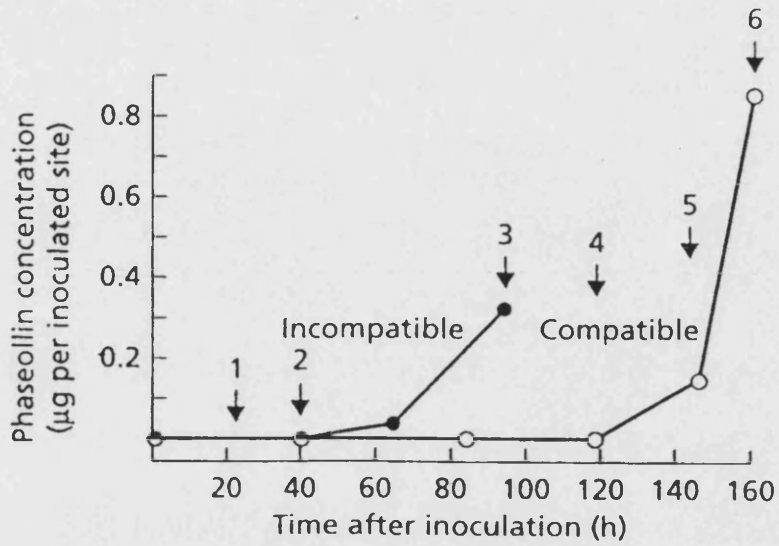

Figure 1.3 Accumulation of phaseollin in French bean inoculated with compatible and incompatible races of the fungus *C. lindemuthianum*. Stage 1, appressorium formation; stage 2, hypersensitive response visible; stage 3, hypersensitive response complete; stage 4, 1% lesions in compatible combination; stage 5, 80% lesions; stage 6, 100% lesions. (data from Bailey and Deverall (1971), diagram from Lucas (1998)).

It seems that production of phytoalexins by susceptible hosts is not an uncommon phenomenon but they are produced too late to stop the pathogen. These findings agree with very early statements made by Müller that the relative rate at which a phytoalexin is produced in a particular host-pathogen combination is probably more important than the final concentration of the compound present (Müller, 1961).

It is difficult to imagine that rapidly dying cells undergoing the HR could mobilise the complex metabolic pathways necessary for the synthesis of phytoalexins. Therefore it is thought that phytoalexins are synthesised in the living cells surrounding the dying cells and are absorbed and accumulate within the hypersensitive cells. This was demonstrated with phaseollin in French bean leaves (Hargreaves and Bailey, 1978). Further experiments showing that living bean cells can metabolise phaseollin (Skip et al., 1977; VanEtten et al., 1982) has led to the following model. Cells undergoing the HR are thought to release elicitors on membrane damage that induce *de novo* synthesis of phytoalexins in neighbouring

cells. These phytoalexins then accumulate in the infected dying cells where fungal growth is inhibited. Any phytoalexin remaining in the living tissues is then metabolised by the plant (Bailey, 1982).

Phytoalexin production is not only thought to be involved in plant defence as part of the HR, it has also been implicated in the resistance of plant species to vascular pathogens. However even this resistance may involve some death by hypersensitivity of invaded root cells (Tjamos and Smith, 1975) and of contact XP cells around challenged vessels (Mace et al., 1976; Cooper et al., 1996). Phytoalexins produced in response to vascular pathogens have been found in many species of plant but have been studied in the most detail in cotton, tomato, elm, alfalfa and most recently in cocoa (Bell and Mace, 1981; Resende et al., 1996). Again both rate and magnitude of phytoalexin synthesis have been correlated with the resistance of cultivars to wilt diseases. In callus cultures derived from near-isogenic lines of tomato cultivars resistant or susceptible to either *Verticillium* or *Fusarium* wilt and in the xylem tissue of the *in planta* interactions, more rapid accumulation of phytoalexins, primarily the terpenoid rishitin, occurred in resistant compared to susceptible tissue (Khatib et al., 1974; Tjamos and Smith, 1974; McCance and Drysdale, 1975; Stromberg and Corden, 1977; Hutson and Smith, 1980; Kroon et al., 1991). In tomato plants inoculated with *F. oxysporum* both root and stem xylem of resistant cultivars contained about twice as much rishitin as susceptible cultivars at 24 and 48h after inoculation. By 72h the concentrations were similar in the cultivars and from then on the concentrations were greatest in the susceptible cultivar increasing with the severity of symptoms (McCance and Drysdale, 1975; Gentile and Matta, 1976). Again this suggests that the relative rate of phytoalexin production is more important in resistance than the final amount of the phytoalexin produced. However, in the same study it was shown that the concentration of rishitin obtained in the resistant plants was not enough to cause inhibition of germination or affect germ tube growth if the phytoalexin was distributed about the tissue evenly. In most cases the accumulation of phytoalexins to inhibitory levels is probably dependent upon a two-component resistance process of localisation and phytoalexin build up (McCance and Drysdale, 1975; Tjamos and Smith, 1975; Conway and MacHardy, 1978; Mace, 1978; Hutson and Smith, 1980; Smith and MacHardy, 1982). The rapid production of vascular occlusions in resistant interactions has been implicated in allowing this localisation and phytoalexin build up to occur within

xylem vessels. Hutson and Smith (1980) used a histochemical test for terpenoids to show that phytoalexins were more concentrated at infection sites of resistant interactions than in susceptible interactions. The combination of physical and chemical resistance mechanisms was based on the sealing off of the infection sites by tyloses thereby blocking the transpiration flow, preventing upward movement of propagules and creating a static environment within the vessels so that phytoalexins could operate efficiently. Furthermore, absorption of phytoalexins by tyloses, gels and gums, and their location in the lumen of the vessel allowed the phytoalexins to be placed directly in the path of the pathogen (Bell and Mace, 1981).

Recently Resende et al. (1996) investigated phytoalexin production by *Theobroma cacao* in response to the vascular wilt fungus *V. dahliae*. Four phytoalexins were identified by nuclear magnetic resonance (NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS), from inoculated stems of resistant cocoa genotypes. The most abundant and polar compound found was the triterpene arjunolic acid. Of intermediate polarity and of least abundance were the related phenolics 3,4-dihydroxyacetophenone and 4-hydroxyacetophenone. Perhaps the most interesting of the phytoalexins discovered and the topic of this research was the least polar compound, unambiguously identified as elemental sulphur (Fig. 1.4). As evidence for its involvement in resistance, elemental sulphur was found to accumulate to fungitoxic levels, as determined by *in vitro* bioassays, in the xylem but not in other tissues of inoculated plants and persisted for more than 60d. Persistence also implied fungitoxicity as many fungi can metabolise sublethal levels of elemental sulphur (Beffa, 1993a)

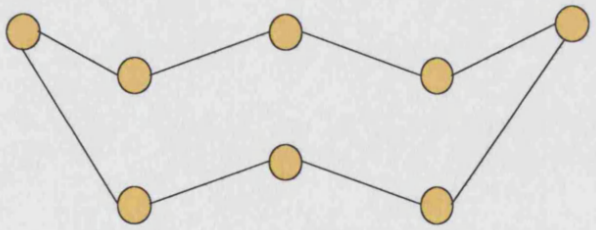

Figure 1.4 Elemental sulphur (S^0). The allotropy of sulphur is far more extensive than any other element except carbon. The most common and most stable allotrope of sulphur is the yellow, orthorhombic α form to which all other modifications revert at room temperature. It contains S_8 molecules in a crown structure and is also known as *cyclo-*S_8 (Greenwood and Earnshaw, 1997, Rossotti, 1998). This is the form of elemental sulphur thought to be produced by resistant *T. cacao* plants in response to *V. dahliae*.

Sulphur afforded the unusual opportunity of cellular localisation of an antimicrobial compound by coupled scanning electron microscopy – energy dispersive X-ray microanalysis (SEM-EDX). This revealed high concentrations of sulphur in scattered contact XP cells, within vessel walls and in gels occluding vessels (Fig. 1.5), areas that may be in direct contact with the xylem invading pathogen (Cooper et al., 1996).

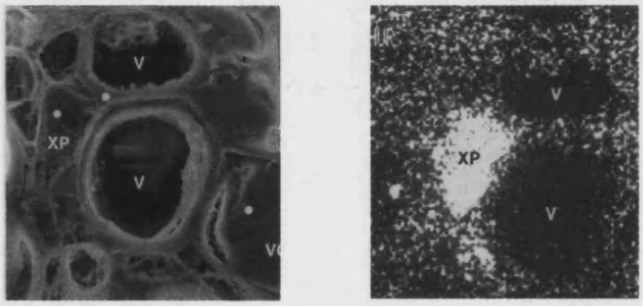

Figure 1.5 Accumulation of sulphur in a contact xylem parenchyma cell of a resistant, *V. dahliae*-inoculated, *T. cacao* plant. The SEM image (a) is a transverse section of vascular tissue from a resistant *T. cacao* plant challenged with *V. dahliae*. The corresponding dot map (b) shows sulphur accumulation in white, in the contact XP cell between two xylem vessels. V, vessel lumen; VG, vascular gel; XP, xylem parenchyma cell (from Cooper et al. (1996)).

It is thought that the presence of elemental sulphur in contact XP cells could reflect accumulation in hypersensitive cells, which lack metabolic capabilities. As described previously the death of scattered XP cells is thought to occur in response to vascular diseases (Tjamos and Smith, 1975; Mace et al., 1976). Therefore sulphur may accumulate to high levels in these necrotic cells after production by adjacent living cells as described for the production of phaseollin in bean leaves responding hypersensitively to *C. lindemuthianum* (Bailey, 1982; Mansfield, 2000). The persistence of elemental sulphur in xylem tissues suggests unavailability to living cells, because wheat and spinach cells can metabolise elemental sulphur in their chloroplasts (Legris-Delaporte et al., 1987; Joyard et al., 1988; Jolivet et al., 1995).

Elemental sulphur formation is a property of many specialised prokaryotes such as photosynthetic and chemoautotrophic bacteria (Schmidt et al., 1987; Visser et al., 1997; Reinartz et al., 1998). There are very few examples of elemental sulphur production by eukaryotes. The first of these discoveries was the detection of elemental sulphur, below that required for toxicity, in the resting structures of

several fungi particularly dormant spores and sclerotinia (Pezet and Pont, 1977). Following this, the production of elemental sulphur was found in two red algae *Erythrophyllum delesserioides* (Izac et al., 1982) and *Ceramium rubrum* (Ikawa et al., 1973). Later a green alga *Chlorella fusca* was also shown to accumulate elemental sulphur from the metabolism of compounds used for growth (Kraus et al., 1984). The characterisation of elemental sulphur from spinach chloroplasts was the first example of elemental sulphur accumulation in a higher plant. Chloroplasts were incubated in the presence of $^{35}SO_4^{2-}$ which resulted in the light dependent formation of a chloroform-soluble, sulphur-containing compound which was later identified as S_8 (Joyard et al., 1988). A report by Kylin et al., (1994) showed the presence of elemental sulphur in the epicuticular wax of several gymnosperms and angiosperms including that of *Pinus sylvestris* needles. At first this was assumed to be of anthropogenic origin from fertilisers, fungicides or industrial emissions but its presence in plants grown in clean air samples suggested that it was of endogenous origin. The positioning of this elemental sulphur and the previous detection of other endogenous fungistatic substances in the epicuticular waxes of pine, led to the suggestion that S^0 may be part of a constitutive antifungal defence system (Kylin et al., 1994). As yet these reports are the only examples of elemental sulphur production by eukaryotes. Its production in resistant *T. cacao* plants in response to *V. dahliae* is the only report of elemental sulphur as an induced antimicrobial compound and of any inorganic element (other than structural functions in cell walls of calcium and silicon) contributing directly to active defence. However this production is by as yet an uncharacterised pathway. It seems ironic that man's probably oldest pesticide (Tweedy, 1981) may also function in this role in living plants.

1.6 The aims of the project

It is possible that the production of elemental sulphur by plants, particularly in response to a challenge by an incompatible pathogen, may be widespread. Previous failure in studies on phytoalexins to detect S_8 may be due to its high hydrophobicity which results in the element coinciding with the solvent front in thin layer chromatography (TLC) bioassays; this zone may have often been ignored because it is associated with impurities that are artifactually fungitoxic and difficult to separate (Joyard et al., 1988). The aim of the following project was firstly to define a method that would allow quantification of elemental sulphur present in biological samples (the method used to identify elemental sulphur in *T. cacao* was

qualitative). A survey of many plant-pathogen interactions would then be undertaken to determine whether the production of elemental sulphur is widespread within the plant kingdom and particularly if it is linked to incompatible responses. Following this, a model system would be chosen and localisation of sulphur at the infection site would again be attempted using SEM-EDX. Furthermore, an attempt would be made to begin the elucidation of the biosynthetic pathway of elemental sulphur production in the model system by investigating the effect of elemental sulphur production on endogenous sulphur metabolism. Finally, an investigation into the toxicity of elemental sulphur to various fungal and bacterial plant pathogens would be undertaken to determine whether the amounts detected in the plants would in fact be toxic to the invading pathogen and if any pathogens specific to elemental sulphur-producing hosts were more tolerant to S^0 than related non-pathogens.

Chapter 2

Quantitative Analysis of Elemental Sulphur from Biological Materials by Gas Chromatography – Mass Spectrometry

2.1 Introduction

In order to investigate further the phenomenon of elemental sulphur as an induced antimicrobial substance and to extend the study to other biological materials, an accurate method for quantitative analysis of elemental sulphur in biological extracts was needed.

In the following chapter a novel GC-MS method for quantification of $^{32}S_8$, utilising isotope dilution analysis of added $^{34}S_8$ standard, is described. Also described for the first time is a previously unreported phenomenon of thermal dissociation and recombination of $^{32}S_8$ and $^{34}S_8$ molecules *via* S_2 species, resulting in mass spectra containing ion clusters representative of atom-level mixing of labelled and unlabelled S_8.

2.2 Materials and Methods

2.2.1 Plant and fungal samples for analysis

Plant and fungal samples to be analysed for elemental sulphur are described in detail in chapter 3.

2.2.2 Sample preparation

Plant samples were comminuted in liquid nitrogen in a pestle and mortar or in a coffee grinder (Gaggia type ML, Milan, Italy) if grinding by hand was impossible. They were then warmed to 0°C and extracted with 10 mL/g fresh wt. dichloromethane (HPLC grade, Fisher, Loughborough, UK) for 10 min to extract non-polar compounds such as lipids, hydrocarbons, fatty acids and elemental sulphur. Internal standard of ^{34}S (90 atom %, Aldrich, Gillingham, UK) made up in dichloromethane (50 ng/μL) was added to the extract at this point. The amount of internal standard added ranged from 5 ng/g fresh wt to 4 μg/g depending on sample type. The proportion added was made as similar as possible to the amount of ^{32}S expected in the sample, as predicted by preliminary experiments. Extracts were filtered through 1PS filter paper (Whatman, Maidstone, UK) into round bottom flasks and the dichloromethane removed by rotary evaporation. The residue was redissolved in 20 mL hexane (HPLC grade, Fisher) and run through an 8 mL Extract Clean™ silica column (prepared from cartridges, frits and silica (60A) obtained from Alltech Associates, Carnforth, UK) to remove any high molecular wt compounds and polar compounds still present in the extract. The column was further eluted with additional hexane (10 mL) and the combined hexane elutants were evaporated. The sample was dissolved in a known volume of dichloromethane (optimised for each type of sample) for analysis by GC-MS.

Verticillium dahliae spores and mycelium were separated from culture fluids by centrifugation (3,000*g*, 10 min). The pellet was then comminuted in dichloromethane (10 mL) by vortexing with glass beads (≤106 μm, Sigma, Poole, UK). The culture fluids were placed in a separation funnel with an equal volume of dichloromethane. The funnel was shaken and allowed to separate twice and the layer containing the dichloromethane collected. Internal standard of ^{34}S was added as above to both samples and the extracts processed by Extract Clean™ silica chromatography as above.

2.2.3 Internal standard and calibration curve production

Calibration of the GC-MS used the ^{34}S and a ^{32}S standard (both 50 ng/μL). From these standards, solutions with defined ratios of ^{32}S : ^{34}S were produced ranging from 1:10 to 5:1 and analysed by GC-MS. The response ratio was calculated for both S_2 and S_8 ions by determining the areas of the peaks for the mass/charge (*m/z*) 272, 256, 68 and 64 ions and calibration curves for S_8 and S_2 were plotted.

2.2.4 GC-MS instrumentation and analysis

All data were obtained using a Hewlett-Packard 5890 gas chromatograph coupled to an HP-5970 mass selective detector (Hewlett Packard Ltd., Winnersh, Berkshire, UK) operated in electron impact ionisation mode. Data were processed using Hewlett-Packard Enhanced ChemStation software (version A.03.00). Samples were chromatographed on a SGE BPX5, 25m x 0.25 mm column (SGE, Milton Keynes, UK). The carrier gas was He at a pressure of 80 kPa. 1μL injections of sample were made manually in cool, on-column mode, thus avoiding initial depolymerization (the S_8 ring is unstable at temperatures >119°C). A retention gap (0.53 mm inside diameter (ID)) of between 2m and 5m length was used to prevent non-volatiles entering the GC column. The column temperature regime adopted was: 35°C for 2 min, rising by 25°C per min to 200°C, and then by 15°C per min to 320°C. The MS source temperature was 150°C and the transfer line temperature was 250°C. The molecular ions of $^{32}S_8$ and $^{34}S_8$ sulphur (*m/z* 256 and 272) as well as the daughter ions (*m/z* 64 and 68) were analysed at 15 cycles per s with a dwell time of 50 ms using the single ion monitoring mode (SIM).

2.2.5 Calculations of S_8 content

The amount of $^{32}S_8$ present in the sample was calculated by comparing the areas of the peaks of the *m/z* 256 and 272 ions followed by reference to the $^{32}S_8$:$^{34}S_8$ calibration curve. This result was confirmed by comparing the areas of the peaks of the *m/z* 64 and 68 ions and calibrating with the $^{32}S_2$:$^{34}S_2$ curve.

2.3 Results

2.3.1 Thermal dissociation and recombination of $^{32}S_8$ and $^{34}S_8$ molecules

Pure standard solutions of ^{32}S and ^{34}S (50 ng/μL) and a 1:1 mixture were analysed by GC-MS on full scan mode in order to determine the amount of thermal dissociation and recombination caused by the high temperatures and subsequent cooling during the GC-MS process (Fig. 2.1). In the pure solutions the $^{32}S_8$ and $^{34}S_8$ rings broke down to the daughter S_2 molecules at the high temperatures as seen by the strong peaks of the *m/z* 64 and 68 ions respectively. Other ions indicative of S_3 (*m/z* 96 and 102), S_4 (*m/z* 128 and 136), S_5 (*m/z* 160 and 170), S_6 (*m/z* 192 and 204), S_7 (*m/z* 224 and 238) and S_8 (*m/z* 256 and 272) could also be noted suggesting partial or complete recombination during cooling. These dissociation and recombination events not only appear to involve S_2 molecules but also individual S atoms in order to produce S_3, S_5 and S_7 molecules. Small peaks to the right of these major peaks can also be noted. These represent other isotopes of sulphur (^{32}S, ^{33}S and ^{34}S) present in the standards showing that they are not completely pure (Fig. 2.1a and b). In the 1:1 mixture of ^{32}S and ^{34}S thermal dissociation and reassociation also occurred, but during recombination compounds containing mixtures of ^{32}S and ^{34}S atoms were produced as well as pure ^{32}S and ^{34}S compounds (Fig. 2.1c(i)). This can been seen most clearly with the S_2 molecules where ions of *m/z* 64 (pure $^{32}S_2$), 68 (pure $^{34}S_2$) and 66 (a $^{34}S^{32}S$ mixed molecule) can be detected (Fig. 2.1c(ii)).

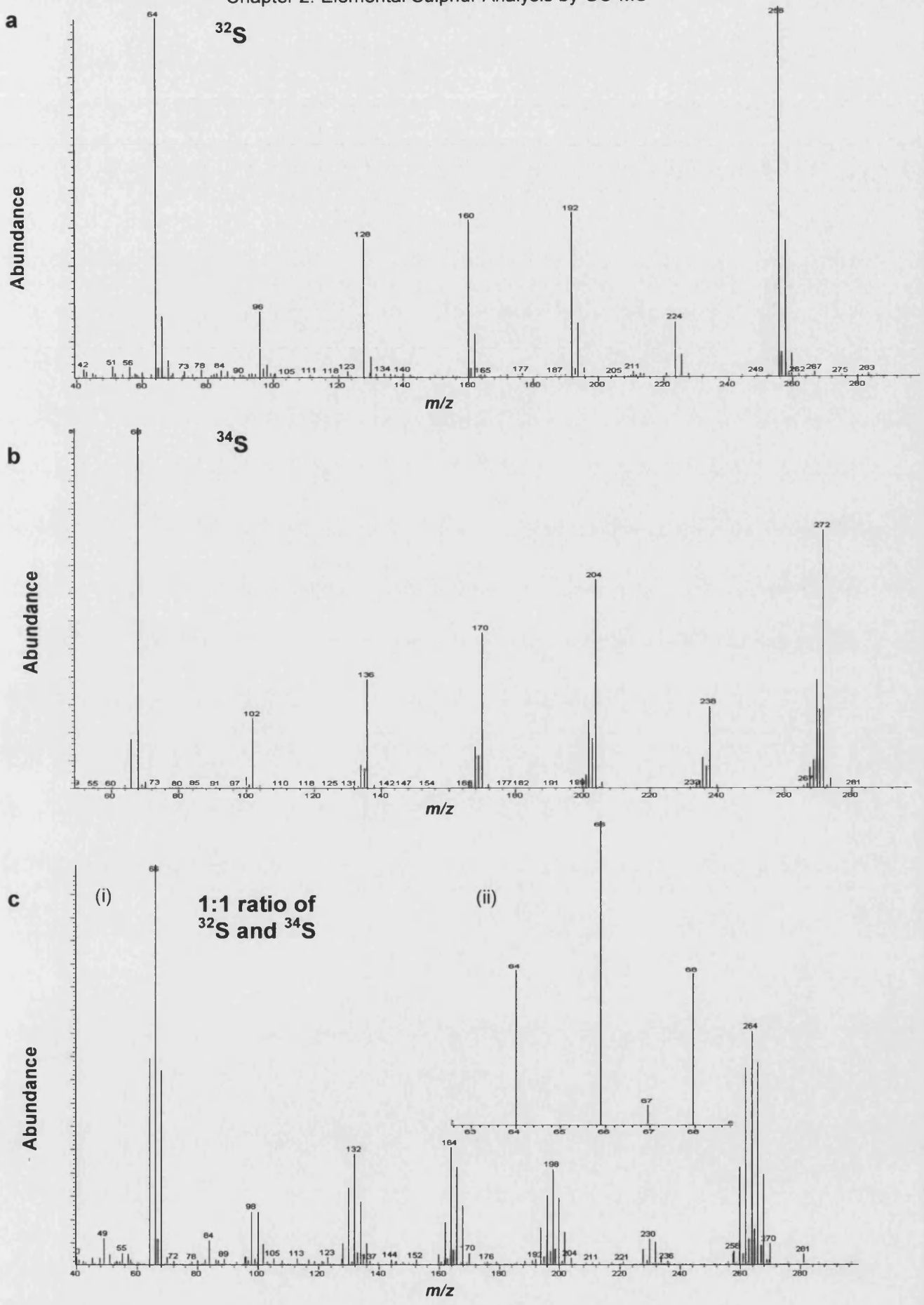

Figure 2.1 GC-MS mass spectra of standard sulphur solutions. Pure standard solutions of ^{32}S (a), ^{34}S (b) and a 1:1 mixture of ^{32}S:^{34}S (c) were analysed by GC-MS on full scan mode. Thermal dissociation and recombination is evident by the presence of ions indicative of S_2, S_3, S_4, S_5, S_6 and S_7 polymorphs in pure standards (a) and (b). Atom level mixing during recombination is seen in the mass spectrum from the 1:1 mixed standard by the presence of a range of different *m/z* ions for each polymorph (ci). Mixing is most apparent with the mixed S_2 molecule that has an *m/z* of 66 indicative of $^{32}S^{34}S$ (cii).

2.3.2 Calibration curves

To allow for dissociation and reassociation when quantifying the amount of ^{32}S present in samples, calibration curves were produced. The calibration curve for S_8 is shown in Fig. 2.2.

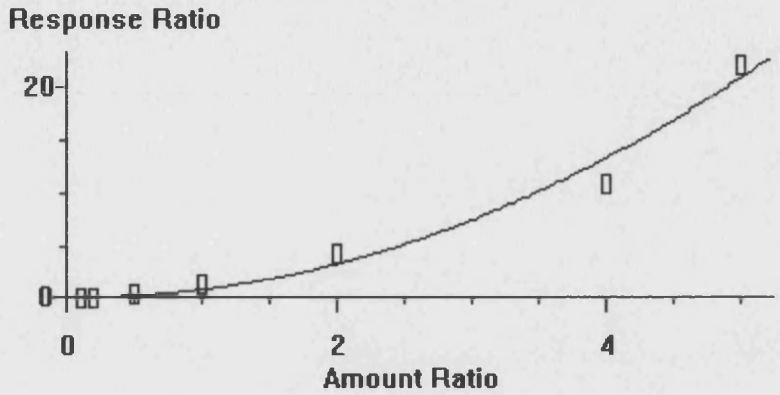

Figure 2.2 Standard response curve for $^{32}S_8$ using $^{34}S_8$ as an internal standard. Solutions containing mixtures of ^{32}S and ^{34}S were analysed by GC-MS in SIM mode. The ratio of isotopes injected was plotted against the response ratio for the 64 and 68 ions to produce an S_2 calibration curve and for the 256 and 272 ions to produce an S_8 calibration curve. The S_8 curve was used preferentially for quantification of elemental sulphur in samples and is shown above. The curve is quadratic and runs through (0,0). The formula for the curve is $\mathbf{R = (8.56 \times 10^{-1}) A^2 - (1.2 \times 10^{-1}) A + 0}$ where A is the amount ratio and R is the response ratio.

2.3.3 Analysis of plant material

As described in more detail in chapter 3, a survey was undertaken in order to determine whether the production of elemental sulphur in response to microbial-challenge was widespread within the plant kingdom. The material harvested, extracted and analysed by GC-MS for this survey reflected the parasitic habit of the fungal or bacterial pathogen i.e. vascular-invading (xylem) or foliar-infecting (leaves) (Table 2.1). The spores and mycelium of the fungus *V. dahliae*, as a representative fungal pathogen, were also tested for elemental sulphur.

Plant	Pathogen	Tissue harvested
Tomato	*Verticillium dahliae* *Ralstonia solanacearum* *Pseudomonas syringae* pv. *phaseolicola*	Xylem Xylem Leaves
Cotton	*Verticillium dahliae*	Xylem
Strawberry	*Verticillium dahliae*	Crowns and Petioles
Tobacco	*Fusarium oxysporum* f. sp. *nicotianae* *Pseudomonas syringae* pv. *tomato*	Xylem Leaves
French Bean	*Fusarium oxysporum* f. sp. *phaseoli* *Pseudomonas syringae* pv. *tomato*	Xylem Leaves
Maize	*Erwinia stewartii*	Leaves
Cabbage / Senna hybrid	*Peronospora parasitica*	Leaves
Cabbage	*Pseudomonas syringae* pv. *tomato*	Leaves
Lettuce	*Pseudomonas syringae* pv. *tomato*	Leaves
Arabidopsis	*Pseudomonas syringae* pv. *tomato*	Leaves
Barley	*Pseudomonas syringae* pv. *tomato*	Leaves
	Verticillium dahliae	Spores and mycelium

Table 2.1 Plant and fungal material harvested, extracted and tested by GC-MS. The table above shows all the plant material used during the survey and analysed successfully for elemental sulphur by GC-MS.

The GC-MS SIM method described in this chapter was found to be suitable for all samples tested to give an accurate quantification of the elemental sulphur present. Analysis of the S_8 ions was always used preferentially for quantification, as the spectra produced by the S_2 ions proved to be greatly affected by other compounds particularly hydrocarbons present in the extract and defined peaks were not always produced. The sensitivity of the GC-MS method varied with the plant material tested. Xylem samples gave fewer background peaks surrounding the sulphur peaks on GC-MS SIM after extraction than leaf samples and consequently sensitivity was greater. Elemental sulphur in xylem samples could be detected as a defined peak at levels above 10 ng/g fresh wt. Elemental sulphur in some leaf samples such as tobacco was also detected at these levels, but for other leaves such as cabbage this method was less sensitive and sulphur could only be detected at levels above 100 ng/g. Variability between GC-MS analyses was assessed by three replicate measurements of the same sample and was found to be ±4%. Fig. 2.3 shows the simultaneous SIM traces from the molecular ions $^{32}S_8$ (*m/z* 256) and $^{34}S_8$ (*m/z* 272) of a sample of tobacco xylem which has produced elemental sulphur as part of a resistance response after infection with the vascular

fungal pathogen *F. oxysporum f. sp. nicotianae*. Details of timing and levels of elemental sulphur present in the other plant-pathogen interactions listed in Table 2.1 are described in the chapter 3.

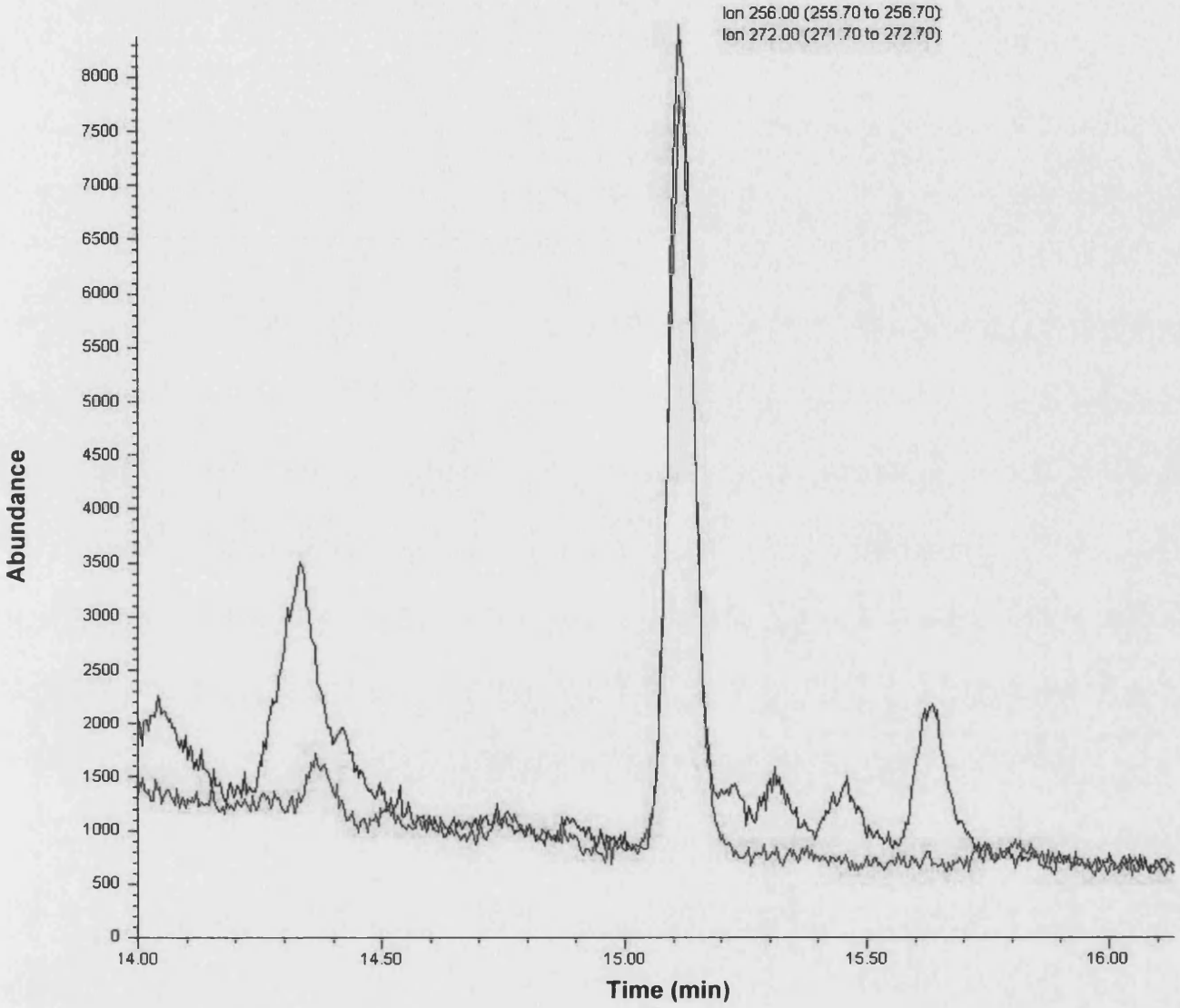

Figure 2.3 GC-MS chromatograms of resistant tobacco xylem challenged with *F. oxysporum* f. sp. *nicotianae*. Plants were harvested at 12 days post-inoculation (dpi), the xylem extracted in organic solvents, 50 ng/g fresh wt ^{34}S standard added and 1μL analysed by GC-MS. The elemental sulphur peak falls at retention time 15.1 min as determined by analysis of standards. Simultaneous SIM traces of the molecular ions *m/z* 256 ($^{32}S_8$) (black trace) and *m/z* 272 ($^{34}S_8$) (pink trace) are shown. Quantification was achieved by comparing the areas under the peaks and by reference to the S_8 calibration curve. The amount of elemental sulphur present in this sample was calculated to be 60.5 ng/g.

2.4 Discussion

Elemental sulphur was detected in *Theobroma cacao* vascular tissue in response to a challenge by *V. dahliae*. It was initially hypothesised that the sulphur was from a sulphur-rich phytoalexin that had broken down to elemental sulphur during extraction and purification, but there was a lack of ^{1}H or ^{13}C NMR signals that would result from a sulphur-containing organic compound. Also, sulphur was consistently extracted with a wide polarity range of solvents and detected as elemental sulphur by TLC without intensive purification. Furthermore, X-ray crystallography and GC-MS in full scan mode were used to identify unambiguously the compound from *T. cacao* as elemental sulphur (Cooper et al., 1996; Resende et al., 1996). However the GC-MS method used to detect this elemental sulphur was not quantitative and required the presence of large amounts of elemental sulphur in order for a positive detection. To determine whether the production of elemental sulphur in response to microbial-challenge was widespread within the plant kingdom and to determine in more detail the kinetics and levels of elemental sulphur accumulation the GC-MS method was improved.

Firstly, a defined amount of ^{34}S was added during the initial stages of extraction to the material to be analysed in order to quantify the amount of ^{32}S present in the sample and to allow for any losses of ^{32}S that might occur during the extraction procedure. This proved to be more complex than originally thought. It was already known that S_8 would dissociate to S_2 and recombine during the GC-MS process as the S_8 ring is unstable at temperatures >119°C. This phenomenon was seen during the analysis of elemental sulphur in *T. cacao* extracts (Cooper et al., 1996; Resende et al., 1996). However, the mass spectra produced from samples that contained both ^{32}S and the ^{34}S standard, showed ion clusters representative of atom-level mixing i.e. compounds containing both ^{32}S and ^{34}S. This was unexpected and appears to be a previously unreported phenomenon not only for elemental sulphur but also for any other element (M. Beale pers. comm., IACR Long Ashton, Bristol, UK). It does allow us to be even more certain that the element in the samples is elemental sulphur as no other compound would be able to interact with ^{34}S in this way. To counteract this problem a S_8 calibration curve was constructed by analyses of solutions containing different ratios of ^{32}S:^{34}S and this was used for quantification of the ^{32}S in the samples. A second alteration to the method is that a SIM mode of GC-MS analysis was used instead of full scan mode. In SIM mode only the *m/z* 64, 68, 256 and 272 ions were monitored instead

of all ions and this allows approximately ten times greater sensitivity as more time is spent analysing each ion (S. Croker pers. comm., IACR Long Ashton, Bristol, UK).

Previously the need to extract and quantify accurately elemental sulphur in biological samples has arisen in many fields of research. Techniques were developed to detect and in some cases quantify elemental sulphur production in the specialised bacteria, fungal resting structures, algae, plant epicuticular waxes and spinach cells mentioned previously in chapter 1 (Ikawa et al., 1973; Pezet and Pont, 1977; Izac et al., 1982; Kraus et al., 1984; Schmidt et al., 1987; Hazeu et al., 1988; Joyard et al., 1988; Steudel et al., 1990; Chan and Suzuki, 1993; Kylin et al., 1994; Visser et al., 1997; Reinartz et al., 1998; Prange et al., 1999). Also elemental sulphur is the active ingredient in many pharmaceutical preparations for the control of acne, the levels of which have to be checked routinely (McLaughlin and Sherma, 1994). Concern for the adverse environmental effects of large-scale combustion of sulphur-containing fossil fuels has led to intense interest in the forms of sulphur found in these fuels, especially coal. Elemental sulphur appears to be formed on the surface of the mineral sulphide, pyrite, a major constituent of coal. Its detection and quantification is therefore important to the coal industry, particularly as its hydrophobicity may be used to separate the pyrite from coal by flotation (Buchanan et al., 1993; Louie et al., 1993; Turcotte et al., 1993; Toniazzo et al., 1999; McGuire and Hamers, 2000). The detection and quantification of elemental sulphur also has significance for the petroleum industry, at sewage treatment works and other industrial processes where highly toxic sulphide is released in the wastewater. Bioreactors or catalysts are being developed in order to convert this sulphide to less toxic elemental sulphur (Stefess et al., 1996; Henshaw et al., 1997; Manova et al., 1997; Visser et al., 1997). In agriculture elemental sulphur is increasingly being added to soils to improve crop yields and an accurate test is required to determine when an application is required (Clark and Lesage, 1989; Jolivet, 1993; Mengel, 1993; P. Williams pers. comm., Wilton Estate, Salisbury, UK).

Techniques previously used to detect and/or quantify elemental sulphur vary widely even within the different areas of research mentioned above. These include; cyanolysis, i.e. the conversion of elemental sulphur to thiocyanate, followed by the addition of Fe^{3+} that is converted by thiocyanate to Fe^{2+}, which is

detected colorimetrically by spectrophotometry (Bartlett and Skoog, 1954; Sörbo, 1957; Hazeu et al., 1988; Chan and Suzuki, 1993; Stefess et al., 1996; Henshaw et al., 1997), gravimetry (Veena and Padma, 1995), voltammetry (Rozan et al., 2000), X-ray photoelectron spectroscopy (Smart et al., 1999; Toniazzo et al., 1999), Raman spectroscopy (Turcotte et al., 1993; Toniazzo et al., 1999), X-ray absorption near edge spectroscopy (Prange et al., 1999), GC coupled to a flame photometric detector or an atomic emission detector (Clark and Lesage, 1989; Louie et al., 1993; Andersson and Holwitt, 1994; Kylin et al., 1994), MS (Krauss et al., 1984; Joyard et al., 1988), MS-MS (Kylin et al., 1994), TLC (Pezet and Pont, 1977; Krauss et al., 1984; Joyard et al., 1988; McLaughlin and Sherma, 1994; Ramsden, 1995), high-performance liquid chromatography (HPLC) (Lauren and Watkinson, 1985; Steudel et al., 1990; Buchanan et al., 1993; Henshaw et al., 1997; McGuire and Hamers, 2000) and GC-MS (Toniazzo et al., 1999).

Some of these methods are not specific to elemental sulphur, for example X-ray photoelectron spectroscopy does not clearly distinguish between sulphide, elemental sulphur and polysulphide and therefore it has to be used in combination with other techniques (Smart et al., 1999; Toniazzo et al., 1999). Some, such as TLC, gravimetry and GC with an atomic emission detector are not sensitive enough for this investigation (Louie et al., 1993; McLaughlin and Sherma, 1994; Veena and Padma, 1995). For others such as Raman spectroscopy and X-ray absorption near edge spectroscopy, quantification would prove difficult (Prange et al., 1999; McGuire and Hamers, 2000).

Quantification requires the use of standards. In all of the techniques above where quantification is used, standards are either external and ^{32}S (McGuire and Hamers, 2000), or internal but a different compound; for example benzothiazole was used as an internal standard in GC with an atomic emission detector (Louie et al., 1993). The use of ^{34}S as an internal standard with GC-MS, as far as the author is aware, has never before been attempted and provides the only completely accurate way of quantifying ^{32}S in the biological samples.

In conclusion GC-MS analysis in SIM mode with an internal standard of ^{34}S provides a sensitive and convenient method of accurately detecting and quantifying ^{32}S present in biological samples.

Chapter 3

Detection of Elemental Sulphur in Compatible and Incompatible Interactions Involving Fungal and Bacterial Vascular and Leaf Diseases

3.1 Introduction

Until recently the formation of elemental sulphur by organisms was thought only to be a property of specialised prokaryotes (Schmidt et al., 1987; Visser et al., 1997; Reinartz et al., 1998) and had only been described in eukaryotes for a few fungi and algae (Ikawa et al., 1973; Pezet and Pont, 1977; Izac et al., 1982; Kraus et al., 1984). It appears that this phenomenon might be more widespread and elemental sulphur could have a frequent role in pathogen resistance. Sulphur could function in preformed defences, as suggested by its occurrence in the cuticular wax of several gymnosperms and angiosperms (Kylin et al., 1994), and in induced resistance, as suggested by its production in resistant genotypes of *Theobroma cacao* in response to *Verticillium dahliae* (Cooper et al., 1996; Resende et al., 1996).

To investigate the theory that elemental sulphur production could be a widespread phenomenon in higher plants and play a role in defence, a survey was undertaken. Plants from various families were challenged with compatible and incompatible, bacterial and fungal, leaf and vascular pathogens to determine whether elemental sulphur was produced and associated with incompatibility. From this survey it was hoped that a model system would be found in order to investigate further the cellular localisation of the sulphur by SEM-EDX and to begin to elucidate the biochemical origin of the elemental sulphur, which is by a pathway(s) previously uncharacterised.

For each incompatible interaction analysed in the survey it is important to know the genetic basis of resistance and its phenotypic expression. Table 3.1 attempts to summarise the literature to date. Although used regularly in the literature for many vascular interactions, the term "tolerance" has been avoided as for none of the interactions used in the survey has tolerance been distinguished from resistance (chapter 1). Instead the term "resistant" is used to describe those vascular interactions where symptoms are either absent or visible only in the lowest leaves

and colonisation is restricted mainly to the roots. "Resistant" is also used to describe those interactions where leaves have been challenged with incompatible pathogens and a HR is induced. The term "intermediate resistant" is used to describe plants that show mild visible disease symptoms in response to vascular pathogens above the lowest leaves and/or where colonisation has been detected routinely in the stems of root-inoculated plants. Furthermore, "intermediate resistant" is used to describe interactions where there is a slight, but not systemic, spread of the pathogen from the point of inoculation in challenged leaves, but that results in a defined lesion. In all cases hosts classed as "resistant" or "intermediate resistant" show less symptoms and colonisation than those plants classed as "susceptible".

The literature provides information from many labs and therefore there are many differences in materials and conditions used such as susceptible and resistant plant cultivars, plant ages on inoculation, pathogen isolates, inoculation methods and environmental conditions (*eg.* moisture, temperature, light intensity, photoperiod, growth substrate). It is important to note that many of the vascular interactions used in the survey are based on multigenic host resistance where a single genotype can give rise to variations in wilt resistance due these differences (Ashworth et al., 1979; Pullman and DeVay, 1982a; Pullman and DeVay, 1982b; Salgado and Schwartz, 1993; Ray et al., 1995; Ben-Yephet and Shtienberg, 1997; Shaw et al., 1997). Monogenically determined resistance although less affected by environmental conditions (Foster and Walker, 1947) may be influenced by inoculation methods, plant age and pathogen virulence. For example, a severe method of inoculation such as severing the taproot and exposing it to a fungal spore suspension, or the use of stem puncture inoculation (Resende et al., 1995), can result in a higher level of colonisation in comparison to natural root infection. This is probably because the roots are thought to serve as a major site for resistance to many vascular pathogens (Garber and Houston, 1967; Gibbins and Wright, 1968; Tsror and Nachmias, 1995).

Host	Pathogen	Genetic basis of resistance	Level of resistance	Related references
Tomato (GCR 218)	*Verticillium dahliae* (Dvd-T5)	Monogenic host resistance (*Ve* gene)	Resistant	Schaible et al. (1951); Cooper and Wood (1980); Diwan et al. (1999)
Tobacco (C9)	*Fusarium oxysporum* f. sp. *nicotianae*	Multigenic host resistance	Intermediate resistant	Mueller and Morgham (1996)
French bean (A55)	*Fusarium oxysporum* f. sp. *phaseoli* (B13)	Multigenic host resistance	Intermediate resistant	Salgado and Schwartz (1993); Salgado et al. (1993) Cross et al. (2000); Fall et al. (2001)
Tomato (Hawai 7996)	*Ralstonia solanacearum* (GMI 1000)	Multigenic host resistance	Intermediate resistant	Grimault and Prior (1993); Prior et al. (1996); Thoquet et al. (1996); McGarvey et al. (1999)
Field Corn (IFSI 90-1)	*Erwinia stewartii*	Host resistance (genetics of this exact interaction are unknown although from analysis of other resistant or intermediate resistant maize lines, resistance is thought to involve between 1 and 4 genes)	Intermediate resistant	Wellhausen (1937); Smith (1971); Blanco et al. (1977); Braun (1990); Meyer et al. (1991); Parker and Hooker (1993); Ming et al. (1999); Brown et al. (2001)
Cotton (Sicala V2)	*Verticillium dahliae* (Australian field isolate)	Multigenic host resistance	Intermediate resistant	Hill et al. (1999) McFadden et al. (2001) Cotton Seed Distributors Variety Guide (2001-2002)
Strawberry (Red Gauntlet)	*Verticillium dahliae* (12008)	Multigenic host resistance	Intermediate resistant	Bringhurst et al. (1968); Shaw et al. (1996)
Cabbage / senna hybrid (OL97049-57)	*Peronospora parasitica* (P006)	Monogenic host resistance	Resistant	Lucas et al. (1988) Leckie et al. (1996)
* *Arabidopsis thaliana* (WS D63)	*Pseudomonas syringae* pv. *tomato* (DC3000)	Monogenic host resistance	Resistant	Hinsch and Staskawicz (1996)
Tomato (GCR 26).	*Pseudomonas syringae* pv. *phaseolicola* (race 6)	Non-host resistance	Resistant	He (1996); Heath (2000a)
Barley (Golden Promise), French bean (The Prince), Lettuce (Suzan), Cabbage (April), Tobacco (Rustica)	*Pseudomonas syringae* pv. *tomato* (DC3000)	Non-host resistance	Resistant	He (1996); Charkowski et al. (1998); Heath (2000a)

Table 3.1 A summary of the incompatible plant pathogen interactions used in a survey to investigate elemental sulphur production as an induced defence response. The genetic basis of resistance for each interaction and its phenotypic expression are stated and referenced to the literature. The references quoted above refer as closely as possible to the interactions used in the survey.

* Since completion of this thesis the author has discovered that the interaction between *Arabidopsis* and *P. syringae* pv. *tomato* DC3000 is compatible. Every further reference to this interaction should be reconsidered regarding the susceptibility of the response. A strain of DC3000 has been modified to carry the *avrRps4* gene causing an incompatible interaction with *Arabidopsis*. This strain would be used for future research (Hinsch and Staskawicz 1996).

3.2 Materials and Methods

3.2.1 Plant Growth

Plant Seed Source

Tomato GCR 26 and GCR 218, tobacco Rustica, lettuce Suzan, French bean The Prince and barley Golden Promise seeds were obtained from the University of Bath (Bath, UK) seed stocks. Tomato Super Marmande and Hawai 7996 seeds were supplied by C. Boucher (INRA, Toulouse, France). Tobacco C9 and 86-4 seeds were provided by J. LaMondia (Connecticut Agricultural Experiment Station, Connecticut, USA). *Arabidopsis* ecotype WS D63 seed was obtained from Nottingham *Arabidopsis* Stock Centre (Nottingham, UK). Cabbage April seed was purchased from Suttons seeds (Torbay, UK). Sweet corn Jubilee and field corn IFSI 90-1 seeds (both *Zea mays*) were provided by J. Pataky (University of Illinois, Illinois, USA). French Bean Olathe (pinto) and A55 (black) seeds were supplied by H. Schwartz (Colorado State University, Colorado, USA).

Plant growth and cultivation

All plants were grown in a glasshouse except for *Arabidopsis,* which was grown in a Fisons 6000G3/THTL growth chamber (Fisons, Loughborough, UK). Plants were either grown in Levingtons compost (Fine grade 2 potted on with Medium grade 2) or a 1:1 sand:perlite mix and were potted up when required. Plants were watered daily and fed three times a week with a full nutrient solution containing; KNO_3 (7 mM), $MgCl_2.6H_2O$ (0.9 mM), KH_2PO_4 (1.0 mM), $Mg(NO_3)_2.6H_2O$ (1.7 mM), NaCl (0.1 mM), ethylenediamine tetra-acetic acid disodium salt (EDTA) FeNa (0.05 mM), $Ca(NO_3)_2.4H_2O$ (5 mM), $CaCl_2.6H_2O$ (2 mM), $CH_3COOZn.2H_2O$ (1.3 µM), H_3BO_3 (24.5 µM), $Cu(NO_3)_2.3H_2O$ (0.6 µM), $(NH_4)_2$ $Mo.4H_2O$ (0.8 µM), FeNa EDTA (50.1 µM), $Mn(NO_3)_2.4H20$ (9.3 µM) and 1.0 mM SO_4^{2-} supplied as $MgSO_4.7H_2O$ (commercial plant foods were found not to contain sulphate). The growth temperature was varied according to plant species and temperature required for pathogen infection. Supplementary illumination was supplied by Phillips 400W high-pressure sodium lamps (Phillips Lighting Ltd., Croydon, UK) for a 16h day length for all plants, with the exception of *Arabidopsis,* which required a 12h day length to promote leaf growth. Biological control (Scarletts Plant Care, Colchester, UK) was introduced when needed against aphids (by *Aphidius*), thrips (by *Amyblyseius cucumeris*), white fly (by *Encarsia formosa*) and sciarid fly (by *Hypoaspis miles*).

3.2.2 Inoculation of plants with fungal vascular pathogens

Source, growth and maintenance of fungal vascular pathogens

Verticillium dahliae, isolate Dvd-T5 race 1 was provided by K. Dobinson (Agriculture and Agri-food, Ontario, Canada). *Fusarium oxysporum* f. sp. *nicotianae* was provided by J. LaMondia. *F. oxysporum* f. sp *phaseoli* isolate B13 was donated by H. Schwartz. All were stored long term as spore suspensions in glycerol (25% v/v) at -70°C. When required 10 μL of the spore suspension was pipetted onto Czapek dox agar medium (appendix A1.1) and incubated at 25°C for two weeks for *V. dahliae* or 4d for *F. oxysporum*.

Preparation of fungal vascular pathogens for inoculation

To produce an inoculum, a shake culture was made in Czapek dox liquid medium at 25°C, 150 rpm. Three d 20 mL starter cultures were produced in 50 mL centrifuge tubes from an agar culture (Bibby Sterilin Ltd., Staffordshire, UK) and used to inoculate fresh 400 mL cultures in 1L conical flasks that were incubated for a further 4d. The resulting spore suspension was filtered through two layers of muslin and centrifuged at 1,000*g* for 10 min. The pellet was resuspended twice in sterile distilled water (pH 6.5). Spore concentration was determined with a haemocytometer and diluted as necessary with sterile distilled water (pH 6.5).

Root-inoculation of tomato plants with *V. dahliae*

Eight week old GCR 26 and GCR 218 tomato plants (near-isogenic lines which are susceptible and resistant respectively to *V. dahliae*) grown in sand or compost, in 22.5 cm pots (Synprodo Plantpak Ltd, Maldon, UK), were root-inoculated by pouring 50 mL of the *V. dahliae* spore suspension (1 x 10^7 spores/mL) onto the growth medium. Control plants were sham-inoculated with 50 mL of sterile distilled water (pH 7). Following inoculation, all plants were watered to distribute the inoculum through the sand or compost. The glasshouse temperature was maintained at 25 ± 3°C and plants were observed daily for symptoms.

Root-inoculation of tobacco plants with *F. oxysporum* f. sp. *nicotianae*

Six week or eight week old 86-4 and C9 tobacco plants (susceptible and intermediate resistant respectively to *F. oxysporum* f. sp. *nicotianae*) grown in compost, in 22.5 cm pots, were root-wounded prior to inoculation in order to facilitate rapid, relatively synchronous infection of the plants. A cylindrical wooden rod of diameter 5 mm was used to stab vertically down through the compost at

four points evenly distributed around the base of the stem and approximately 3 cm from the stem. 150 mL of a *F. oxysporum* spore suspension (4 x 10^6 spores/mL) was poured onto the compost. Control plants were sham-inoculated with 150 mL of sterile distilled water (pH 7). Following inoculation, all plants were watered to distribute the inoculum through the compost. The glasshouse temperature was maintained at 27 ± 2°C and plants were observed daily for symptoms.

Root-inoculation of French bean plants with *F. oxysporum* f. sp. *phaseoli*

Three week old Olathe (pinto) and four week old A55 (black) French bean plants (susceptible and intermediate resistant respectively to *F. oxysporum* f. sp. *phaseoli*) grown in compost, in 3L pots (Aeroplas UK Ltd, Tipton, UK) were root-wounded as described above. Different cultivars grew at different rates and therefore plants of different ages were used to synchronise the number of nodes present on inoculation. 200 mL of a *F. oxysporum* spore suspension (2 x 10^7 spores/mL) in an initial experiment or 90 mL of a *F. oxysporum* spore suspension (7.5 x 10^6 spores/mL) in a later experiment was poured onto the compost. Control plants were sham-inoculated with sterile distilled water (pH 7). Following inoculation, all plants were watered to distribute the inoculum through the compost. During inoculation the glasshouse temperature was maintained at 21 ± 2°C as plants inoculated on hot, sunny days were found by Salgado and Schwartz (1993) and in this study to suffer leaf scalding. The following day the temperature was raised to 28 ± 2°C by day and 21 ± 2°C by night (Salgado and Schwartz, 1993) and plants were observed daily for symptoms.

3.2.3 Inoculation of plants with bacterial vascular pathogens

Source, growth and maintenance of bacterial vascular pathogens

Ralstonia solanacearum isolate GMI 1000 was provided by C. Boucher. *Erwinia stewartii* was provided on infected maize leaves by J. Pataky. *E. stewartii* was isolated from infected leaves by surface sterilising in 10% sodium hypochlorite solution for 2 min and washing twice in sterile distilled water. Leaves were then comminuted in a sterile pestle and mortar with 1 mL of sterile distilled water (pH 7). The resulting suspension was diluted in a ten fold dilution series with sterile distilled water to 10^{-4} and 50 µL spread onto nutrient yeast glycerol agar (NYGA) (appendix A1.2) to give single colonies. A single colony was lifted and restreaked

onto NYGA to complete the reisolation. Both pathogens were stored long term in glycerol (25% v/v) at –70°C. When required *R. solanacearum* was streaked out onto bacto-agar glucose triphenyl-tetrazolium chloride (BGT) agar (appendix A1.2) and incubated at 30°C for 2d. *E. stewartii* was streaked out onto NYGA and incubated at 25°C for 2d.

Preparation of bacterial vascular pathogens for inoculation

Highly mucoid individual colonies of *R. solanacearum* or *E. stewartii* were lifted and transferred to 400 mL bacto-peptone (B) broth (appendix A1.2) or nutrient yeast glycerol broth (NYGB) (appendix A1.2) in 1L volumetric flasks respectively. Flasks were incubated overnight at 150 rpm at 30°C or 25°C respectively to produce a turbid suspension of cells. This suspension was centrifuged at 1,000*g* for 20 min, the pellet resuspended twice in sterile distilled water (pH 7) and then diluted to give an optical density (OD) at A_{600} of 0.6 corresponding to 8 x 10^8 cfu/mL for *R. solanacearum* and an OD at A_{600} of 0.5 corresponding to 2.6 x 10^8 cfu/mL for *E. stewartii.* Inoculum concentration for *R. solanacearum* was initially estimated by using a *Pseudomonas syringae* OD/viable count curve (Fig. 3.2) and that for *E. stewartii* was estimated from an *Erwinia amylovora* OD/viable count curve (Youle 1989). Both were more accurately quantified by performing a viable count on the inoculum used to infect the plants.

Root-inoculation of tomato plants with *R. solanacearum*

Eight week old Super Marmande and Hawai 7996 tomato plants (susceptible and intermediate resistant respectively to *R. solanacearum*) in 22.5 cm pots, grown in compost, were root wounded as explained above and 450 mL of the bacterial suspension was poured onto the compost. Control plants were sham-inoculated with sterile distilled water (pH 7). Following inoculation, all plants were watered to distribute the inoculum through the compost. The glasshouse temperature was maintained at 30°C ± 2°C by day and 27°C ± 2°C by night and plants were observed daily for symptoms.

Leaf-inoculation of maize plants with *E. stewartii*

Six week old sweet corn Jubilee and field corn hybrid IFSI 90-1 (susceptible and intermediate resistant respectively to *E. stewartii*) in 22.5 cm pots, grown in compost were inoculated on leaves 4 and 5 with *E. stewartii* using a "clapper", a

device used to simulate natural infection by the corn flea beetle (the overwintering host of the bacterium) (Fig. 3.1). Control plants were inoculated in the same way but with sterile distilled water (pH 7). The glasshouse temperature was maintained at 26°C ± 2°C and plants were observed daily for symptoms.

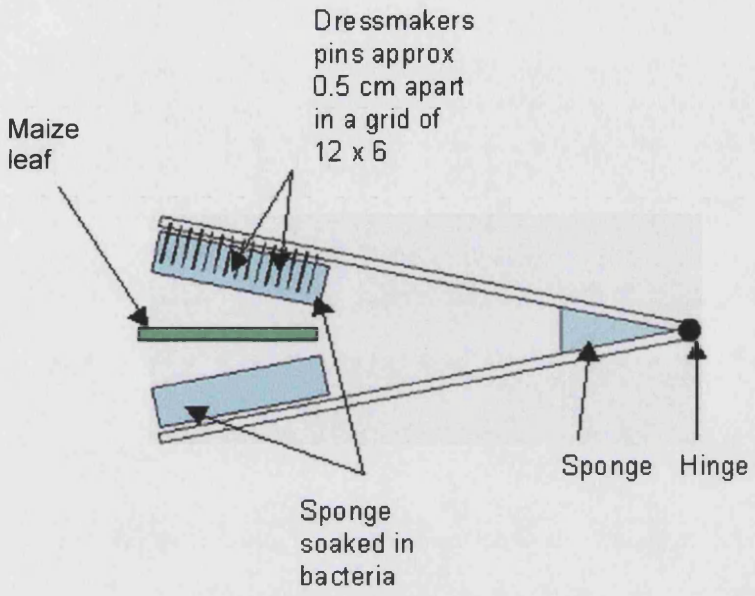

Figure 3.1 The "clapper" used to inoculate maize plants with *E. stewartii* (adapted from Blanco et al. (1977)). Leaves were inoculated by placing one end between the *E. stewartii* soaked sponges and pressure applied causing wounding by the pins and inoculation of the wounds by the bacteria. This was repeated at 2 cm intervals along the leaf.

3.2.4 Inoculation of plants with bacterial leaf pathogens

Source, growth and maintenance of bacterial leaf pathogens

Pathogenic strains of *P. syringae* pv. *phaseolicola* race 6 and *P. syringae* pv. *tomato* DC3000 were supplied by J. Mansfield (Imperial College at Wye, Ashford, UK). Both were stored long term in glycerol (25% v/v) at –70°C. When required stocks were streaked out onto NYGA and incubated at 25°C for 2d to produce single colonies.

Preparation of bacterial leaf pathogens for inoculation

Individual colonies were lifted, transferred to 20 mL NYGB in 50 mL centrifuge tubes and incubated overnight at 25°C, 150 rpm to produce a turbid suspension of cells. The bacterial suspensions were then centrifuged at 1,000*g* for 20 min and the pellets resuspended twice in sterile distilled water (pH 7). These suspensions were then diluted to an OD of 0.5 at A_{600} corresponding to 1.4×10^8 cfu per mL (an

OD/viable count curve for *P. syringae* was constructed in order to quantify number of viable cells present in the inoculum (Fig. 3.2)).

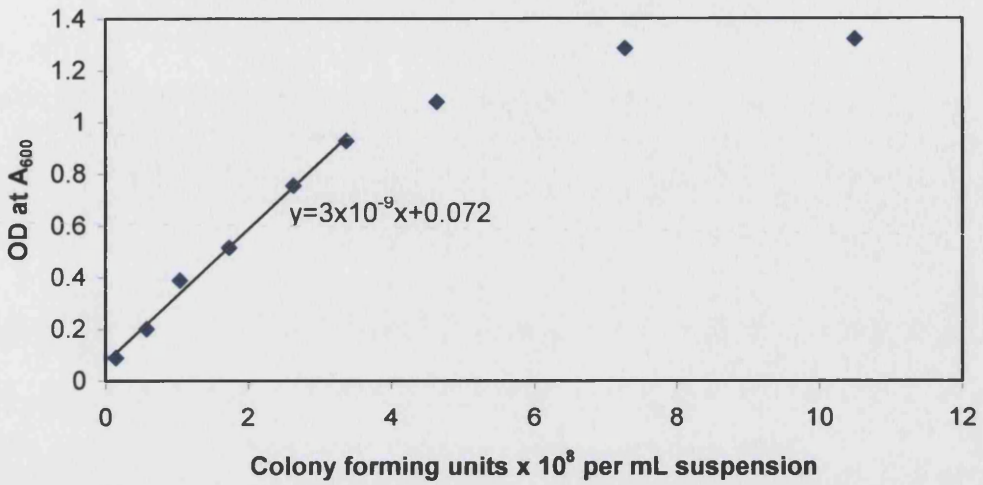

Figure 3.2 Relationship between optical density (A_{600}) and colony forming units in a suspension of *P. syringae* pv. *tomato*. Points represent the number of viable colonies suspended in sterile distilled water (pH 7) present at a defined OD against a sterile distilled water blank. Values were determined by the mean of three replicate viable counts on each suspension. OD is directly proportional to the number of viable cells (Beer-Lambert Law) up to OD 1 and the formula for this relationship is given by $y=3x10^{-9}x+0.072$ (where y=OD at A_{600} and x=cfu/mL).

Leaf infiltration of plants with *P. syringae* pathovars

Two week old barley Golden Promise, three week old French bean The Prince, five week old tomato GCR 26, six week old lettuce Suzan, seven week old cabbage April and eight week old tobacco Rustica, all compost grown, were leaf-infiltrated with an inoculum of an incompatible *P. syringae* pathovar in order to induce hypersensitive lesions. For tomato plants *P. syringae* pv. *phaseolicola* race 6 was used and for all other plants *P. syringae* pv. *tomato* isolate DC3000 was used. A syringe barrel (Terumo, Leuven, Belgium) loaded with the inoculum was applied to the underside of the leaves and pressure applied. Infiltration was evident by water-soaking of interveinal areas. Each infiltration was of approximately standard size for each individual set of plants (1 cm diameter for barley, French bean, cabbage and tomato and 2 cm for lettuce and tobacco). A space of 0.5 to 1 cm was left between one infiltration and the next, and infiltrations continued to be applied until the whole leaf was covered in a series of water-soaked areas. The number of leaves inoculated per plant varied between species;

for barley and bean two leaves were inoculated, for lettuce, tobacco and cabbage three leaves were inoculated and for tomato three leaflets were inoculated on each of three leaves per plant. The glasshouse temperature was maintained at 22°C ± 2°C.

Six week old *Arabidopsis* plants were also leaf-infiltrated with a similar inoculum of *P. syringae* pv. *tomato*. Two infiltrations of 1 cm diameter were made in each leaf, one either side of the midrib. The growth chamber temperature was maintained at 20°C ± 1°C.

Controls for all of these infiltration experiments were inoculated in the same way as the pathogen-inoculated plants but with sterile distilled water (pH 7). Inoculated leaves were marked with a small needle puncture at the leaf tip. A number of plants were also left uninoculated. Plants were observed daily for symptoms.

3.2.5 Reisolation and quantification of *V. dahliae* from the stems of tomato plants following root-inoculation

A direct reisolation technique was used to recover *V. dahliae* from different stem positions (internodes 1, 8 and 15) of GCR 26 and GCR 218 plants root-inoculated with either *V. dahliae* or sterile distilled water. This was done at three time points (13, 20 and 28 days post-inoculation (dpi)). At each time point, three replicate pathogen-inoculated and control plants from both lines were harvested. A section, 4 cm in length, was excised from each of the stated internodes from each plant. All sections were surface sterilised in 100 mL of 10% sodium hypochlorite solution and two drops of Tween 20 for 5 min and then rinsed in two washes of sterile distilled water. Three complete transverse sections, each of thickness 3 mm were cut from the centre of each 4 cm section and plated onto Czapek dox agar containing 50 µg/mL streptomycin. Plates were incubated at 25°C for 6d in the dark and then examined visually for characteristic growth of *Verticillium*. If growth occurred from any of the three transverse sections a positive result was given for that internode. Any growth that was questionable in origin was transferred to Czapek dox agar and incubated for a further 6d at 25°C in the dark for comparison against the wild type isolate of *V. dahliae.*

Quantitative colonisation analysis was performed on the material remaining from the above analysis. Thin transverse sections of individual vascular bundles were

cut with a razor blade from each of the stated internodes of the three replicate inoculated and control plants and examined by light microscopy at 400X magnification. Percentage of xylem vessels infected with fungal hyphae and percentage of vessels occluded with gels or tyloses was calculated (Cooper and Wood, 1980).

3.2.6 Dissection and storage of plant material in preparation for extraction of elemental sulphur

Following inoculation, all plants were left to develop disease symptoms or produce a resistant response. At defined intervals plants were harvested and tissues tested for elemental sulphur accumulation. Prior to each harvest all equipment for harvesting was soaked in dichloromethane to remove any elemental sulphur that may be present.

Dissection of stem vascular tissue from tomato plants following root-inoculation with *V. dahliae* or *R. solanacearum*

V. dahliae-inoculated tomato plants grown in compost were initially left for one of three time intervals, corresponding with those used for assessment of colonisation (13, 20 and 28 dpi), for preliminary quantitative analysis of elemental sulphur. At each interval five plants from each cultivar and from both pathogen-inoculated and control treatments were harvested, the xylem extracted and the material pooled for elemental sulphur analysis. The experiment was later repeated but using plants grown in sand culture to ensure a defined sulphate nutritional regime. This was carried out by S. Hall at IACR Rothamsted (Harpenden, UK) where the facilities for growing plants in sand culture are well established. Three plants were harvested from each cultivar and treatment at 7, 14 and 21 dpi and extracted as described in chapter 2 to produce one sample per plant. Samples were then sent to the University of Bath for elemental sulphur analysis.

R. solanacearum-inoculated tomato plants were harvested at 7 and 14 dpi. At each time point three pooled samples each containing material from three plants were harvested for each cultivar and from both water-inoculated and pathogen-inoculated plants.

For both interactions plants were harvested in the same way. The lower half of the stem up to node eight was removed from each plant, the xylem tissue excised by

scraping away the epidermis with a scalpel and the vascular bundles dissected out with forceps. All xylem tissue was frozen at -70°C for later extraction and elemental sulphur analysis.

Dissection of stem vascular tissue from tobacco plants following root-inoculation with *F. oxysporum* f. sp. *nicotianae*

Tobacco plants inoculated with *F. oxysporum* and water-inoculated controls were left for either 12 dpi in an initial experiment for preliminary quantitative analysis of elemental sulphur with six week old plants or 17 dpi and 35 dpi in a second experiment with eight week old plants. In the initial experiment whole stems were excised from three plants, the xylem removed and the material pooled for each cultivar and treatment. In the second experiment stems were excised only up to node three (xylem containing vascular tissue was far more substantial in older plants) and the xylem collected to give three pooled samples each containing xylem from three plants for each cultivar and treatment at each time point.

To remove the xylem from the surrounding stem tissue the epidermis was peeled away by hand, the stems cut longitudinally and the pith removed with a cork borer. All xylem tissue was frozen at -70°C for later extraction and elemental sulphur analysis.

Dissection of stem vascular tissue from French bean plants following root inoculation with *F. oxysporum* f. sp. *phaseoli*

French bean plants inoculated with *F. oxysporum* and water-inoculated controls were left for 16 dpi in an initial experiment for preliminary quantitative analysis of elemental sulphur and 13 dpi and 22 dpi in a second experiment. In the initial experiment three plants were harvested, the xylem removed and the material pooled for each cultivar and treatment. In the second experiment three pooled samples each containing xylem from five plants were harvested for each cultivar and treatment at each time point.

In both experiments the stem up to node five was excised and the epidermis peeled away by hand. The pith was not removed as it was negligible in comparison to the amount of xylem. All xylem tissue was frozen at -70°C for later extraction and elemental sulphur analysis.

Removal of leaf material from plants challenged with *P. syringae*

In initial experiments, control leaves and leaves challenged with *P. syringae* were harvested at 7 dpi except those of *Arabidopsis* that were harvested at 4 dpi. In later experiments leaves were harvested at 1, 3 and 5 dpi for all except *Arabidopsis* that were harvested at 1 and 5 dpi only due to space limitation in the growth chamber. For tobacco, French bean, cabbage and tomato three pooled leaf samples each from five plants were harvested, for lettuce three pooled samples each from four plants were harvested, for barley one pooled sample from ten plants was harvested and for *Arabidopsis* three pooled samples each from 24 plants were harvested. Leaves were excised from uninoculated, water-inoculated and *P. syringae*-inoculated plants for analysis. In a further experiment involving tobacco and *P. syringae*, hypersensitive lesions were removed from the leaves with a 3 mm, green non-necrotic border. All excised material was washed in sterile distilled water and stored at -70°C for later extraction and elemental sulphur analysis.

Removal of leaf material from plants challenged with *E. stewartii*

Maize plants were harvested at 14 dpi. Four plants were harvested from each cultivar and treatment and the material washed in sterile distilled water, pooled and stored at -70°C for later extraction and elemental sulphur analysis.

Preparation of *V. dahliae* for elemental sulphur analysis

To test whether fungi could produce elemental sulphur a shake culture of *V. dahliae* was produced. Three d starter cultures were produced in 50 mL centrifuge tubes from an agar culture and used to inoculate each of two 250 mL conical flasks containing 100 mL Czapek dox liquid medium that were incubated for a further 3d. The spores and mycelium were separated from the culture fluids and each of these was extracted and analysed for elemental sulphur as previously described (chapter 2).

3.2.7 Material sent from other sources

Cotton plants stem-puncture inoculated with *V. dahliae*

Cotton samples were provided by H. McFadden (CSIRO, Canberra, Australia). CS50 and Sicala V2 (commercial cotton varieties, susceptible and intermediate resistant respectively to *V. dahliae*) were grown in steam-sterilised soil (50% potting mix, 50% Pryor's mix with osmocote) in the glasshouse.

V. dahliae was derived from cultures of a field isolate by S. Allen (Australia Cotton Research Institute, Narrabri, New South Wales, Australia). The fungus was grown on Czapek dox agar for two weeks and conidia harvested by flooding the plates with water. The concentration of spores was obtained with a haemocytometer and adjusted to either 1 x 10^6 conidia/mL or 2 x 10^6 conidia/mL.

At four weeks plants were inoculated twice by placing 20 μL of the conidial suspension on the stem approximately 1.5 cm above the cotyledonary node. Uptake of the inoculum into the transpiration stream was achieved by piercing the stem under the droplet with a 16 mm syringe needle. Control plants were inoculated in the same way but with water. The glasshouse temperature was maintained at 25°C by day and 15°C by night with natural light conditions.

In an initial experiment where cotton plants were inoculated with 2 x 10^6 conidia/mL, plants were harvested at 14 dpi. Stems were harvested from 10 plants and pooled for each cultivar and treatment. In a later experiment where plants were inoculated with 1 x 10^6 conidia/mL, plants were harvested at both 8 dpi and 15 dpi. Three pooled samples each from 13 plants were harvested for each cultivar and treatment. In both experiments the stem was excised from half way up the first node (to avoid the inoculation site) to node four. The epidermis was removed and the remaining tissue collected and stored at -80°C (the pith was not removed as it was minimal in comparison to the xylem tissue). Samples were then lyophilised and dispatched to the University of Bath where they remained in a desiccator with silica gel (Merck, Poole, UK) for later extraction and elemental sulphur analysis.

Strawberry plants root-inoculated with *V. dahliae*

Strawberry samples were provided by A. Soares (HRI, East Malling, UK). Hapil and Red Gauntlet strawberry plants (susceptible and intermediate resistant respectively to *V. dahliae*) were vegetatively propagated into soil and grown in the glasshouse.

V. dahliae isolate 12008 (previously isolated from strawberry) was grown on prune lactose yeast agar (PLYA) (appendix A1.1) at 22°C for two weeks. To produce an inoculum a shake culture was made in prune lactose yeast (PLY) liquid medium (appendix A1.1) at room temperature, 150 rpm for 7d. The resulting suspension

was filtered through two layers of muslin and centrifuged at 16,000*g* for 10 min. The pellet was resuspended in sterile distilled water. Spore concentration was determined with a haemocytometer and adjusted to 1 x 10^6 spores/mL.

Plants were inoculated by a root-dip inoculation method. At four weeks old plants were de-potted, the roots washed in tap water and the tip of the roots removed. The root was then placed in the fungal-inoculum for 5 min and re-potted. Control plants were treated in the same way but dipped into sterile distilled water. The glasshouse temperature was maintained at 22°C with a 14h day length. Plants were harvested at 21 dpi. The petioles were removed and the epidermis peeled away under a dissecting microscope. Crowns were also excised and their epidermis and pith removed. Plants continued to be harvested until 10g of each tissue was collected (*ca.* 20 plants). The material was immediately frozen in liquid nitrogen and stored and transported to the University of Bath at -70°C for later extraction and elemental sulphur analysis.

Brassica oleracea cotyledons inoculated with *Peronospora parasitica*

B. oleracea OL97049-57 (a hybrid of cabbage and senna) samples were provided by J. Byrne (HRI, Wellesbourne, UK). Plants resistant to *P. parasitica* were grown in compost covered in vermiculite in a glasshouse. *P. parasitica,* accession P006, was maintained on susceptible *B. oleracea* seedlings.

When required an inoculum was produced by washing infected *B. oleracea* leaves in sterile distilled water to collect spores. The resulting suspension of sporangia was centrifuged at 1,000*g* for 10 min and the pellet resuspended in sterile distilled water to wash the spores. The spore concentration was determined with a haemocytometer and diluted to 5 x 10^4 spores/mL.

Nine d old plants were inoculated by placing 10 μL of the *P. parasitica* spore suspension onto each cotyledon. Control plants were drop-inoculated with 10 μL of sterile water. Conditions were maintained at 15°C with a 12h photoperiod.

Plants were harvested by removing the cotyledons from 240 *P. parasitica*-inoculated and water-inoculated plants and freezing them in liquid nitrogen. Material was stored and transported to the University of Bath at -70°C for later extraction and elemental sulphur analysis.

3.2.8 Elemental sulphur detection by GC-MS

Sample preparation, addition of ^{34}S internal standard, GC-MS instrumentation and analysis and subsequent calculation of ^{32}S present in samples is detailed in chapter 2.

3.2.9 Localisation of sulphur by coupled SEM-EDX

Localisation of sulphur in tomato stem sections root-inoculated with *V. dahliae*

Transverse and longitudinal sections of thickness *ca.* 2 mm of individual vascular bundles were excised with a razor blade (dichloromethane washed) from the first internode of *V. dahliae*-inoculated and control, susceptible and resistant plants at 12 to 16 dpi (analysis of all treatments could not be completed in 1d and samples could not be stored) and at 28 dpi for cryofixation. Samples were attached to a holder with Tissue Tek and colloidal graphite, plunged into nitrogen slush and sublimed. A 20 nm coating of aluminium was evaporated onto samples (gold obscured the sulphur peak during X- ray analysis and carbon gave images with excessive charging and surface contamination) under vacuum and the samples viewed in a JSM-6310 SEM (JEOL, Peabody, Massachussetts, USA). X-ray analysis was by an AN10000 Energy Dispersive X-ray Analyser (Oxford Instruments Ltd., Marlow, UK).

Lyophilised samples were also prepared at 14 dpi and 28 dpi from all treatments as cryofixed samples could not be stored. Samples were plunged into liquid nitrogen for 5 min, and then transferred under liquid nitrogen to aluminium carriers for lyophilization for 12h. Samples were then mounted on carbon adhesive discs, which were adhered to aluminium planchettes (Agar Scientific Ltd, Stansted, UK). Mounted samples were carbon coated in an E12E Vacuum Coating Unit (Edwards High Vacuum Ltd., Crawley, UK). SEM and X-ray analysis was performed as above.

Two replicate plants were used for cryofixation and a further two for lyophilization for each treatment at each time point. For analysis of relative sulphur levels present at 12 to 16 dpi, cryofixed material was used and 25 vascular areas were randomly chosen from two randomly selected sections from each plant. Localisation of sulphur in the form of dot maps was also performed on these areas as well as in lyophilised sections at 14 dpi and with both cryofixed and lyophilised sections at 28 dpi.

Attempted localisation of sulphur in tobacco leaves showing a HR to *P. syringae* and in uninoculated *Arabidopsis* leaves

P. syringae induced hypersensitive lesions with a 3 mm non-necrotic border and water inoculated segments excised from tobacco leaves, were placed in a holder with "Tissue Tek" and colloidal graphite and plunged into nitrogen slush, as were uninoculated *Arabidopsis* leaves. The leaf samples were then fractured under vacuum in such a way that in samples of challenged tobacco a progression was evident from healthy to necrotic hypersensitive cells. Samples were then sublimed, coated and viewed as previously described for cryofixed tomato stem samples. Two uninoculated leaves were analysed from each of two *Arabidopsis* plants, and two hypersensitive lesions and two water-inoculated areas were analysed from each of two tobacco plants.

3.3 Results

3.3.1 Elemental sulphur analysis in plants inoculated with fungal vascular pathogens

3.3.1.1 Tomato vs. *V. dahliae*

Colonisation of tomato plants by *V. dahliae* and resulting disease symptoms

Symptoms became apparent in infected disease susceptible (GCR 26) tomato plants at approximately 10 to 13 dpi. Plants expressed symptoms of water stress (flaccidity of petioles and leaves) around midday but recovered by evening through to early morning. Epinasty of lower petioles was also apparent at this time (Fig. 3.3). In the following week wilt symptoms became irreversible and severe. Flaccidity, chlorosis and necrosis of the lower leaves progressed to successive leaves up the plant, adventitious roots were produced and by 21 dpi plants were severely wilted and stunted. Resistant (GCR 218) plants had chlorotic areas on the lowest leaves, whilst other parts of the plant appeared healthy and they were a similar height to control plants. Removal of the stem epidermis of susceptible infected plants revealed brown discoloration of underlying vascular bundles in contrast to the cream-coloured xylem tissues of healthy and resistant plants.

(a) (b)

Figure 3.3 Initial symptoms expressed by susceptible (GCR 26) tomato plants inoculated with *V. dahliae* at 14 dpi. Susceptible tomato plants root-inoculated with *V. dahliae* (a) expressed symptoms of water stress, epinasty, stunting of growth and the start of chlorosis particularly in the lower leaves in comparison to the water-inoculated control plants (b) at 14 dpi. These symptoms later progressed to successive leaves up the plant. Note, resistant (GCR 218) tomato plants challenged with *V. dahliae* were indistinguishable from controls at this stage. This is the second time point at which xylem was analysed for elemental sulphur.

Rapid, acropetal hyphal colonisation occurred in infected susceptible stems as shown by both qualitative reisolation and microscopic analysis of percentage of vessels containing hyphae. Colonisation progressed from 5% of vessels infected at internode 1 at 13 dpi (when initial symptoms were evident) to 57% at 28 dpi (Fig. 3.4). Reisolation was positive at all time points for this internode (Table 3.2). Colonisation of internode 8 was slow initially and no vessel colonisation was evident microscopically at internode 15 up to 20 dpi. However reisolation was positive at 20 dpi for two out of three plants at internode 15 suggesting a small number of vessels were colonised at this time. Invasion then progressed rapidly at both internode 8 and 15 to reach around 30% of vessels at 28 dpi and positive reisolation from all plants. In GCR 218 plants, hyphal colonisation by *V. dahliae* was sparse. Only approximately 0.3% of vessels contained hyphae in internode 1 at 13 dpi and hyphae were not detected in this or in higher internodes ≥20 dpi. Reisolation was positive at internode 1 at 13 dpi attributed to the small number of colonised vessels detected. However one out of three plants gave a positive reisolation at internodes 1 and 8 at 28 dpi. Control plants showed no hyphal colonisation or reisolation.

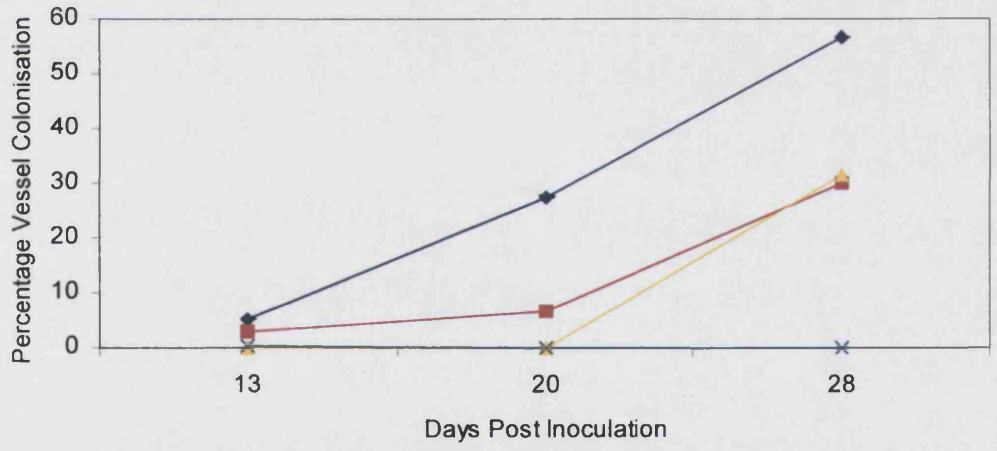

Figure 3.4 Colonisation of susceptible (GCR 26) and resistant (GCR 218) tomato plants inoculated with *V. dahliae*. Analyses were performed at internodes 1 (♦), 8 (■) and 15 (▲) in susceptible plants and internode 1 (x) in resistant plants at 13, 20 and 28 dpi. Transverse sections of individual vascular bundles were cut from each internode of three replicate plants and percentage of vessels colonised was calculated. Points represent percentage of xylem vessels infected with fungal hyphae. Colonisation for GCR 218 was only detectable at 0.3% in internode 1 at 13 dpi and so further data points for other internodes from the resistant variety are omitted for clarity. Chi-square tests at the 95% confidence level revealed significantly higher colonisation of susceptible plants compared to resistant plants at internodes 1 and 8 at 13 and 20 dpi and in all internodes at 28 dpi.

	Susceptible			Resistant		
Days post-inoculation	**Internode 1**	**Internode 8**	**Internode 15**	**Internode 1**	**Internode 8**	**Internode 15**
13	+ + +	+ - -	- - -	? - +	- - -	- ? -
20	+ + +	+ + +	+ - +	- - -	- - -	- - ?
28	+ + +	+ + +	+ + +	- - +	- - +	- - -

Table 3.2 Reisolation of *V. dahliae* from pathogen-inoculated resistant (GCR 218) and susceptible (GCR 26) tomato plants. Three complete transverse sections were excised from internodes 1, 8 and 15 of three replicate plants at 13, 20 and 28 dpi and plated out onto Czapek dox containing streptomycin. Plates were incubated at 25°C for 6d and scored for *V. dahliae* growth. A positive result (+) was given if reisolation occurred in any of the three transverse sections. A negative (-) result was given if none of the three transverse section showed reisolation and a ? was given if the plate was too contaminated to determine a result.

Percentage of vessels containing vascular occlusions (tyloses and gels) was also significantly higher (as determined by chi-square) in vascular tissues of *V. dahliae*-inoculated resistant plants than of susceptible plants until 28 dpi when a similar number of tyloses were present only at internode 1 of both treatments. Control plants showed no vascular occlusion (Fig. 3.5).

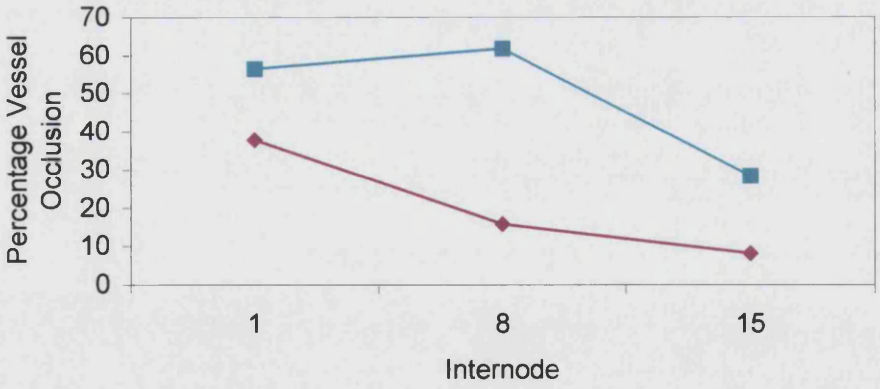

Figure 3.5 Vascular occlusion of susceptible (GCR 26) and resistant (GCR 218) tomato plants inoculated with *V. dahliae* at 20 dpi. Transverse sections of individual vascular bundles were cut from each internode of three replicate susceptible (♦) and resistant (■) plants and percentage of vessels occluded was calculated and plotted. Chi-square tests at the 95% confidence level revealed a significantly higher number of occlusions in the *V. dahliae*-inoculated resistant plants compared to susceptible plants at all internodes at this time point. Analysis at 28 dpi revealed that a similar number of occlusions was present in both *V. dahliae*-inoculated susceptible and resistant plants at internode 1 but the number remained significantly higher at internodes 8 and 15 in the resistant plants (data not shown for clarity).

GC-MS analysis of S_8 in xylem from susceptible and resistant tomato lines inoculated with *V. dahliae*

Following root-inoculation, susceptible and resistant *V. dahliae*-inoculated and control plants were left for one of three time intervals corresponding with those used for assessment of colonisation (13, 20 and 28 dpi) for preliminary quantitative analysis of elemental sulphur. Sulphur was present in both resistant and susceptible *V. dahliae* challenged xylem at all time points. Levels in resistant plants reached a maximum of *ca.* 3 μg/g but in the susceptible only reached a maximum of *ca.* 1 μg/g. No elemental sulphur accumulated in the xylem of control plants. Therefore the experiment was repeated but using plants grown in sand culture to ensure a defined sulphate nutritional regime and the plants were harvested at 7, 14 and 21 dpi for elemental sulphur.

Xylem from control plants did not accumulate elemental sulphur and none was detected in *V. dahliae*-inoculated plants at 7 dpi (before stem colonisation). Subsequently, inoculated susceptible plants showed a slow increase in S_8 reaching 1.88 ± 0.71 μg/g at 21 dpi. In comparison, inoculated resistant plants showed a rapid and more substantial increase in elemental sulphur and contained 10.4 ± 1.7 μg/g at 21 dpi (Fig. 3.6).

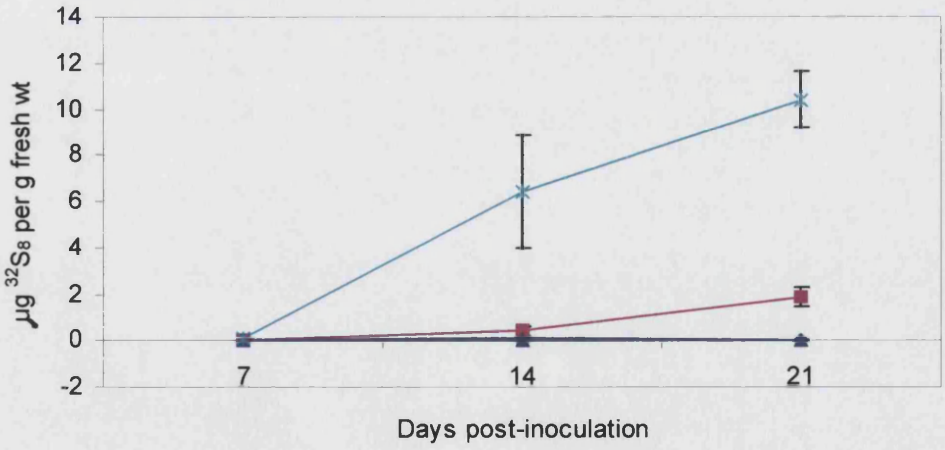

Figure 3.6 GC-MS analysis for S_8 of xylem tissue from resistant and susceptible tomato plants inoculated with *V. dahliae*. Xylem was harvested from three replicate control (♦) and pathogen-inoculated (■) susceptible plants and control (▲) and pathogen-inoculated (x) resistant plants at 7, 14 and 21 dpi for extraction and analysis by GC-MS. Values represent the mean with standard error (SE).

SEM-EDX localisation of sulphur in vascular tissue of tomato plants inoculated with *V. dahliae*

Based on extent of colonisation and the GC-MS analysis above, cryofixed and lyophilised, transverse and longitudinal sections were analysed from the first internode of the tomato stems between 12 to 16 dpi and at 28 dpi to compare relative sulphur levels and tissue and cellular distribution of sulphur in resistant and susceptible, pathogen-inoculated and control plants. Cryofixed samples were coated in aluminium and lyophilised samples were coated in carbon prior to viewing by SEM. At 12 to 16 dpi general area analyses covering 25 vascular areas were made for each cryofixed treatment by X-ray analysis for the detection of sulphur. Sulphur levels were recorded as "high" when the sulphur peak was greater than 50% of the height of the potassium peak, which was always the predominant, endogenous element. Wherever a high level of sulphur was detected, X-ray mapping was performed to enable visualisation of any localised accumulations in the form of a dot map. These were compared to a secondary electron image to determine in which structure the sulphur accumulation had occurred. Further localisation studies were also attempted on lyophilised sections at 14 dpi and cryofixed and lyophilised samples at 28 dpi.

At 12 to 16 dpi only very low levels of sulphur were detected in all areas analysed from control plants (Fig. 3.7(ai) and (aii)). In *V. dahliae*-inoculated, resistant plants 18 out of 25 vascular areas examined showed high sulphur (Fig. 3.7(di) and (dii)) but in the pith cells only very low levels, equivalent to that in control plants, were present (Fig. 3.7(bi) and (bii)). In inoculated susceptible plants, sulphur was low in the majority of vascular areas (17 out of 25) (Fig. 3.7(ci) and (cii)). For localisation studies, cryofixed and lyophilised samples gave similar results. Because cryofixed samples could not be stored, lyophilised samples were used for subsequent analyses. In the vascular areas tested, from both resistant and susceptible stems at 12 to 16 dpi and at 28 dpi, those that had shown "low" sulphur had no accumulations of sulphur above background signal (Fig. 3.8(ai), (aii) and (aiii)) but in those that had shown "high" sulphur, sulphur had accumulated over much of the vascular tissue as evident from comparison with the background signal (Fig. 3.8(bi), (bii) and (biii)). Occasionally, more intense localisations of sulphur were detected in distinct XP cells (Fig. 3.8(ci) and (cii)), gels (Fig. 3.8(di) and (dii)), tyloses and vessel walls, in comparison to lower but still "high" levels detected in surrounding vascular structures.

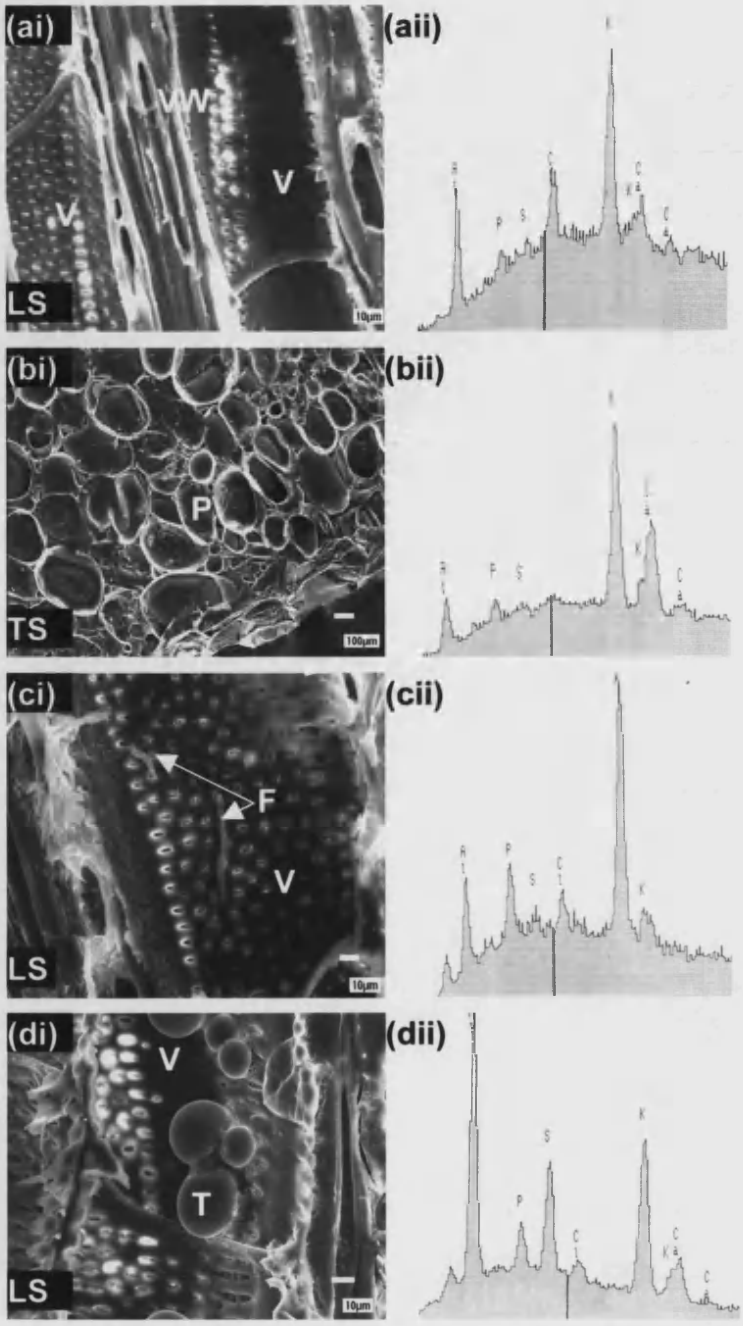

Figure 3.7 Relative sulphur levels in resistant and susceptible *V. dahliae*-inoculated and control stems of tomato plants. Transverse and longitudinal sections from the base of the stem were analysed at 12 to 16 dpi for the detection of "high" (>50% of K peak) or "low" (<50% of K peak) sulphur. 25 area X-ray analyses were made for each treatment and a representative scanning electron image (i) and spectrum from that image (ii) is shown (a to d). Very low levels of sulphur were detected in all areas of control stems analysed (ai) & (aii) and in the central pith cells from resistant pathogen-inoculated plants (bi) & (bii). In susceptible, inoculated plants most vascular areas contained low sulphur (ci) & (cii) in comparison to resistant, inoculated plants where the majority of vascular areas showed high sulphur (di) & (dii). There were more vascular occlusions (gels and tyloses (di)) evident in the resistant vascular tissues at this time than in the susceptible line in which many vessels contained fungal hyphae (ci). Note the aluminium peak derives from the coating evaporated onto the sample. V, vessel lumen; VW, vessel wall; F, fungal hypha; T, tylose; P, stem pith cell; XP, xylem parenchyma cell; VG, vascular gel; TS, transverse section; LS, longitudinal section.

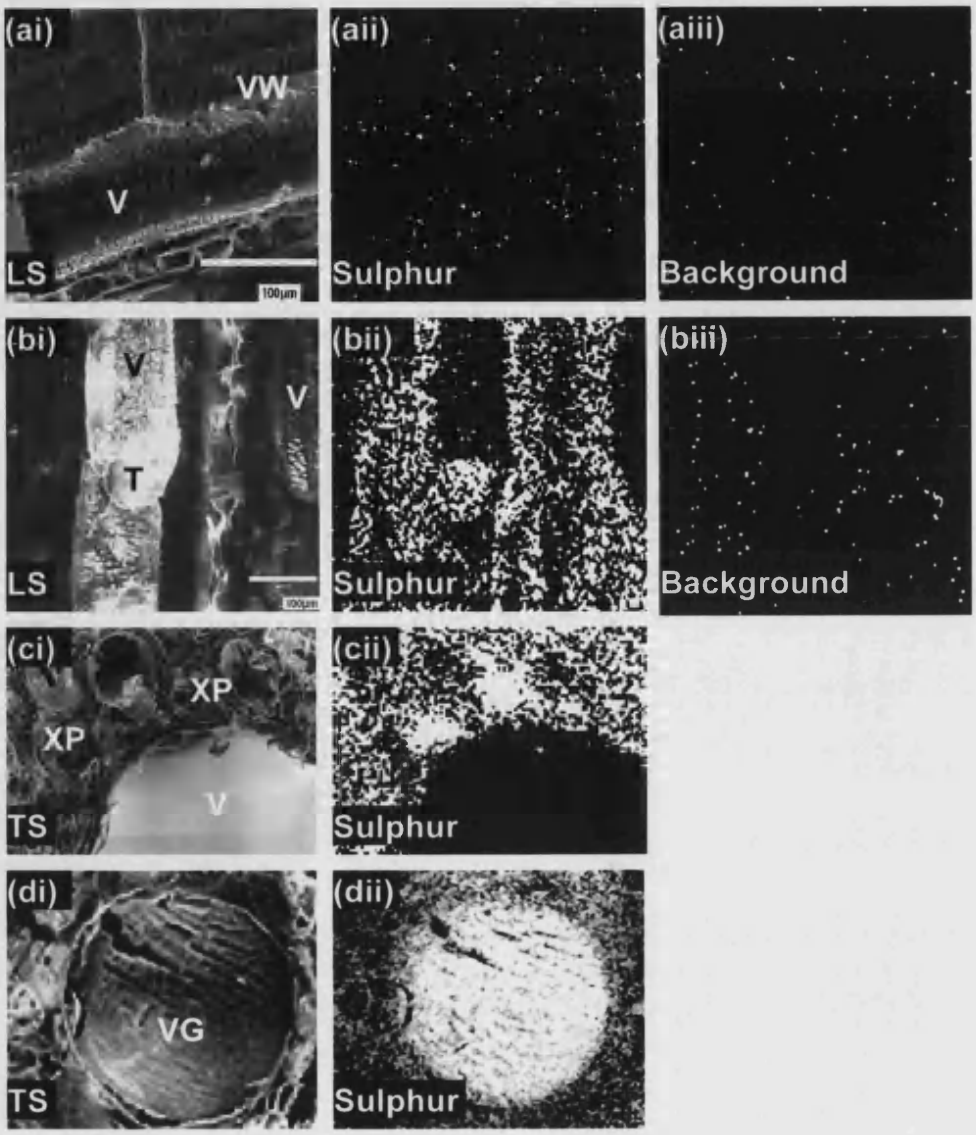

Figure 3.8 Distribution of sulphur in vascular tissues of *V. dahliae*-inoculated and control stems of tomato plants. Transverse and longitudinal sections from the base of the stem were analysed at 12 to 16 and 28 dpi for the detection of "high" (> 50% of K peak) or "low" (<50% of K peak) sulphur. Wherever "high" sulphur was detected an X-ray dot map was produced for localisation of sulphur. Representative SEM images are shown (i) with corresponding dot maps for sulphur (ii) and background noise from the analyser (iii) (a to d). No accumulations of sulphur were found in control samples above background (ai), (aii) & (aiii). In most samples that had high sulphur, sulphur was present over most of the vascular tissue in comparison to background signal (bi), (bii) & (biii). Note the accumulation of sulphur in the tylose (bii). Occasionally there were more intense spots of sulphur in certain structures such as XP cells (ci) & (cii) and gels (di) & (dii). For abbreviations see Fig. 3.7 legend.

3.3.1.2 Cotton vs. *V. dahliae*

Disease symptoms (assessed by H. McFadden)

Disease symptoms became evident in *V. dahliae*-inoculated susceptible plants (CS50) at around 8 dpi as chlorosis followed by necrosis of the lowest leaves that progressed to higher leaves with time. Symptoms also became evident on the intermediate resistant cultivar (Sicala V2) but occurred much later (around 14 dpi) and were much less severe. Symptoms were assessed more precisely on the days of harvesting. Disease severity was based on the degree of leaf necrosis where leaf abscission or complete necrosis was rated 5 and lack of discernible symptoms rated as 0. The first 5 leaves (from the base of the plant) were scored and an overall score for each plant calculated where

$$\text{Plant score} = 1/5 \sum_{1}^{5} \text{Leaf score}$$

In the initial experiment where cotton plants were inoculated with 2 x 10^6 spores/mL, the disease index at 14 dpi for *V. dahliae*-inoculated susceptible plants was 3.2 while the disease index for inoculated intermediate resistant plants was 1.8. Control plants had a disease rating of zero. In the later experiment using an inoculum of 1 x 10^6 spores/mL symptoms were only apparent on the very lowest leaves of *V. dahliae*-inoculated susceptible plants as small areas of necrosis at 8 dpi. No symptoms were evident on inoculated intermediate resistant plants. A disease rating was not calculated at this time. However at 15 dpi when symptoms had progressed, the pathogen-inoculated susceptible plants were given a rating of 2.46 ± 0.16 (Fig. 3.9) and the inoculated intermediate resistant plants were given a rating of 0.82 ± 0.08. These scores were found to be significantly different using the t test ($P=10^{-13}$).

(a) (b)

Figure 3.9 Symptoms expressed by susceptible (CS50) cotton plants inoculated with *V. dahliae* at 15 dpi. Susceptible cotton plants stem-puncture inoculated with *V. dahliae* expressed moderate symptoms at 15 dpi with necrosis and chlorosis having progressed from lower to upper leaves (a). These plants were also stunted in comparison to water-inoculated controls (b). Note, intermediate resistant (Sicala V2) plants inoculated with *V. dahliae* had small areas of chlorosis on the lowest leaves at this time point. This is the second time point at which xylem was analysed for elemental sulphur analysis.

GC-MS analysis of S_8 in xylem from susceptible and intermediate resistant cotton lines

In the initial experiment on cotton vascular tissue harvested at 14 dpi (moderate symptoms on susceptible plants, mild symptoms on the lowest leaves of intermediate resistant plants), "low" levels of elemental sulphur (i.e. the peaks corresponding to sulphur were no higher than the surrounding background peaks on the GC-MS spectrum and so were unlikely to actually be elemental sulphur (P. Gaskin pers. comm., IACR Long Ashton, Bristol, UK)) were detected in control vascular tissue. The vascular tissue from susceptible *V. dahliae*-inoculated plants had accumulated 19 ng/g of S_8 and the tissue from intermediate resistant inoculated plants had accumulated 108 ng/g. However only one sample per cultivar and treatment was tested and so the experiment was repeated to give three samples. Plants were harvested at 8 dpi (initial symptoms in susceptible plants, no symptoms in intermediate resistant plants) and 16 dpi (moderate symptoms in susceptible plants, very mild symptoms in intermediate resistant plants). Again "low" levels of elemental sulphur were detected in controls across both time points. At 8 dpi the vascular tissue from susceptible *V. dahliae*-

inoculated plants showed a small increase to 39.6 ± 2.13 ng/g which was increased to 53.5 ± 2.77 ng/g at 16 dpi. There was a more rapid and substantial increase of elemental sulphur in the vascular tissue from intermediate resistant inoculated plants with an accumulation of 104 ± 15.8 ng/g at 8 dpi which increased to 175.8 ± 20.2 ng/g by 16 dpi (Fig. 3.10).

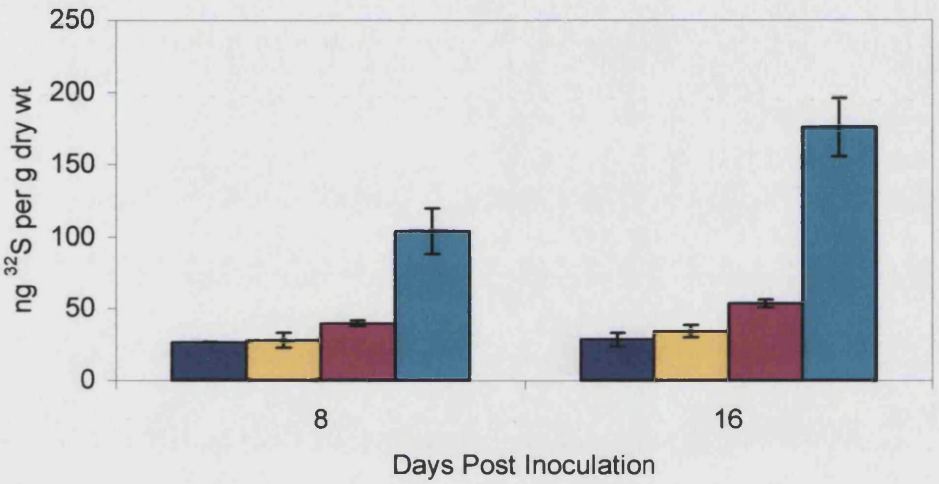

Figure 3.10 GC-MS analysis for S_8 of xylem tissue from intermediate resistant and susceptible cotton plants inoculated with *V. dahliae*. Three pooled samples from each of thirteen control (■) and pathogen-inoculated (■) susceptible plants and control (■) and pathogen-inoculated (■) resistant plants were harvested at 8 dpi (initial symptoms in susceptible plants, no symptoms in intermediate resistant plants) and 16 dpi (moderate symptoms in susceptible plants, mild symptoms in intermediate resistant plants) for extraction and analysis by GC-MS. Values represent the mean with SE.

3.3.1.3 Strawberry vs. *V. dahliae*

Disease symptoms (assessed by A. Soares)

Disease symptoms became apparent in susceptible strawberry plants (Hapil) inoculated with *V. dahliae* at approximately 2 to 3 weeks post-inoculation with sudden symptoms of water stress in outer (older) leaves. Stunting of the plants was also evident in comparison to controls. These water stressed leaves then developed necrosis at the base of their petioles, close to the crown and soon became entirely necrotic (Fig. 3.11) The same symptoms then progressed to younger leaves and by 5 weeks post-inoculation Hapil plants were dead. Intermediate resistant *V. dahliae*-inoculated strawberry plants (Redgauntlet) showed stunting of plants in comparison to controls at 3 weeks post-inoculation. Necrosis became evident at around 5 weeks post-inoculation and due to the

severity of the inoculation method and the level of inoculum Redgauntlet plants died at 8 weeks.

(a) (b)

Figure 3.11 Symptoms expressed by susceptible (Hapil) strawberry plants inoculated with *V. dahliae* at 21 dpi. Susceptible strawberry plants root dip-inoculated with *V. dahliae* expressed moderate symptoms at 21 dpi (a) with necrotic outer older leaves and petioles. These plants were also stunted in comparison to water-inoculated controls (b). Note intermediate resistant (Redgauntlet) plants inoculated with *V. dahliae* also showed stunting at this time point but no leaf necrosis. This is the time point at which xylem was analysed for elemental sulphur.

GC-MS analysis of S_8 in xylem from susceptible and intermediate resistant strawberry lines

In an initial experiment on strawberry petioles and crowns harvested at 21 dpi (moderate symptoms in susceptible plants, mild symptoms in intermediate resistant plants) similar and "low" levels of elemental sulphur were detected in both control and pathogen-inoculated, susceptible and intermediate resistant samples. The experiment was not repeated.

3.3.1.4 Tobacco vs. *F. oxysporum* f. sp. *nicotianae*

Disease symptoms

Disease progression in this interaction was dependent on the age of the plant when inoculated. Susceptible (86-4) plants inoculated with *F. oxysporum* f. sp. *nicotianae* at six weeks of age exhibited initial symptoms at around 8 dpi. The lowest leaves showed signs of water stress and patches of chlorosis, which rapidly became necrotic. Removal of the stem epidermis of susceptible plants at this

stage revealed streaks of brown discoloration of underlying vascular tissue in contrast to the cream coloured vascular tissue in pathogen-inoculated intermediate resistant and control plants. The symptoms spread acropetally until all leaves were either severely chlorotic or totally necrotic at 12 dpi. At this point the vascular tissue was uniformly discoloured and plants were stunted in comparison to controls and intermediate resistant inoculated plants. Susceptible plants inoculated with *F. oxysporum* at eight weeks of age showed initial symptoms on the lowest leaves at 17 dpi. Disease progression was also delayed and severe symptoms (all leaves affected, total vascular browning and stunting) were not evident until 35 dpi (Figs. 3.12 and 3.13).

In both experiments intermediate resistant (C9) plants inoculated with *F. oxysporum* developed chlorosis and in some cases necrosis only on the lowest leaves, whilst other parts of the plant appeared healthy and they were otherwise indistinguishable from controls.

(a) (b)

Figure 3.12 Symptoms expressed by susceptible (86-4) tobacco plants inoculated with *F. oxysporum* f. sp. *nicotianae* at 35 dpi. Susceptible tobacco plants root-inoculated with *F. oxysporum* f. sp. *nicotianae* expressed severe symptoms at 35 dpi (a) with all leaves either necrotic or chlorotic. These plants were also stunted in comparison to water-inoculated controls (b). Note, intermediate resistant (C9) plants inoculated with the same pathogen showed some chlorosis or necrosis at this time but only on the lowest leaves. This is the second time point at which xylem was analysed for elemental sulphur.

(a)

(b)

(c)

Figure 3.13 Vascular browning of xylem tissue extracted from the lower stem of susceptible (86-4) tobacco plants inoculated with *F. oxysporum* f. sp. *nicotianae* at 35 dpi. Susceptible tobacco plants root-inoculated with *F. oxysporum* f. sp. *nicotianae* developed severe vascular browning in the infected xylem tissue (b). Both control xylem (a) and intermediate resistant xylem challenged with *F. oxysporum* (c) remained mainly cream in colour at the macroscopic level but microscopically vascular browning could be detected in gels, tyloses and xylem vessel walls in intermediate resistant xylem challenged with *F. oxysporum*.

GC-MS analysis of S_8 in xylem from susceptible and intermediate resistant tobacco lines

In the initial experiment with six week old tobacco plants harvested at 12 dpi (severe symptoms in susceptible plants, mild symptoms on the lowest leaves of intermediate resistant plants) vascular tissue from susceptible and intermediate resistant control plants contained "low" levels of elemental sulphur. Susceptible *F. oxysporum*-inoculated plants accumulated 30.5 ng/g of S_8 and intermediate resistant inoculated plants accumulated 60.5 ng/g of S_8. The experiment was then repeated on eight week old plants in order to give more vascular tissue per plant and three samples per treatment. Plants were harvested at 17 dpi (initial symptoms in susceptible plants, no symptoms in intermediate resistant plants) and 35 dpi (severe symptoms in susceptible plants, mild symptoms on the lowest leaves of intermediate resistant plants). Susceptible and intermediate resistant control plants again showed "low" levels of elemental sulphur at both time points. *F. oxysporum*-inoculated susceptible plants contained 129.1 ± 41.5 ng/g S_8 in the vascular tissue at 17 dpi that increased to 182 ± 25.3 ng/g at 35 dpi. Inoculated

intermediate resistant plants showed a more rapid and substantial increase in elemental sulphur at 17 dpi reaching 249.5 ± 21.5 ng/g which declined at 35 dpi to 183.7 ± 61.5 ng/g (Fig. 3.14).

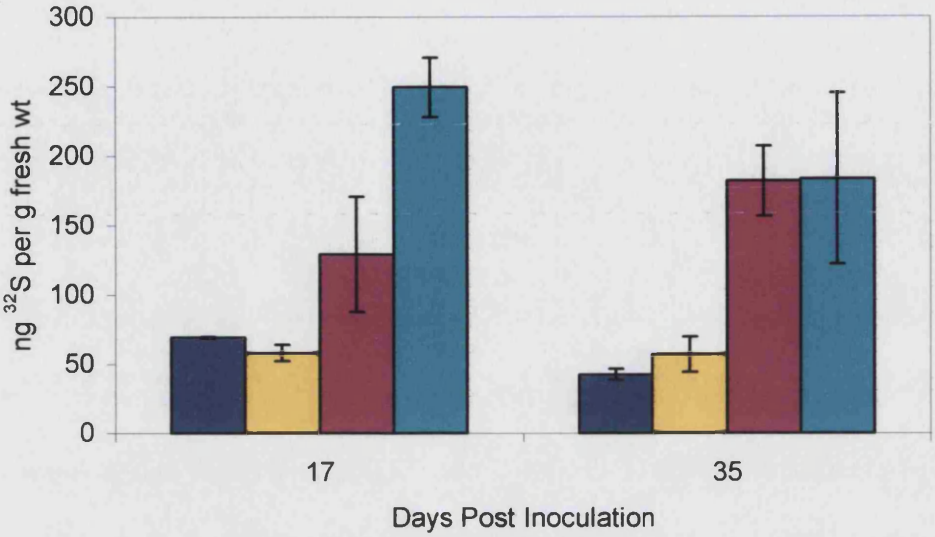

Figure 3.14 GC-MS analysis for S_8 of xylem tissue from intermediate resistant and susceptible tobacco plants inoculated with *F. oxysporum* f. sp. *nicotianae*. Three pooled samples of xylem each from three control (■) and pathogen-inoculated (■) susceptible plants, and control (■) and pathogen-inoculated (■) intermediate resistant plants were harvested at 17 dpi (initial symptoms in susceptible plants, no symptoms in intermediate resistant plants) and 35 dpi (severe symptoms in susceptible plants, mild symptoms on lowest leaves of intermediate resistant plants) for extraction and analysis by GC-MS. Values represent the mean with SE.

3.3.1.5 French bean vs. *F. oxysporum* f. sp. *phaseoli*

Disease symptoms

Symptoms became apparent in susceptible (Olathe) *F. oxysporum* f. sp. *phaseoli*-inoculated French bean plants at 13 dpi and were very similar to those seen in susceptible tobacco plants inoculated with *F. oxysporum*; the lowest leaves were water stressed and chlorotic and vascular browning was evident on removal of stem epidermis. At 22 dpi all leaves were either severely chlorotic or necrotic, there was total vascular browning and plants were stunted in comparison to controls and pathogen-inoculated intermediate resistant plants (Fig. 3.15). Intermediate resistant (A55) plants inoculated with *F. oxysporum* developed chlorosis on the lowest leaves and some plants showed some streaks of vascular browning at the base of the stem at 22 dpi but otherwise were indistinguishable from controls.

Figure 3.15 Symptoms expressed by susceptible (Olathe) French bean plants inoculated with *F. oxysporum* f. sp. *phaseoli* at 22 dpi. Susceptible bean plants root-inoculated with *F. oxysporum* f. sp. *phaseoli* expressed severe symptoms at 22 dpi with all leaves severely necrotic or chlorotic. These plants were also stunted in comparison to water-inoculated controls. Note, intermediate resistant (A55) plants inoculated with the same pathogen showed some chlorosis or necrosis at this time but only on the lowest leaves. This is the second time point at which xylem was analysed for elemental sulphur.

GC-MS analysis of S_8 in xylem from susceptible and intermediate resistant French bean lines

In the initial experiment with French bean, plants were harvested at 16 dpi (moderate symptoms in susceptible plants, very mild symptoms on the lowest leaves of intermediate resistant plants). Neither the control nor *F. oxysporum*-inoculated plants accumulated elemental sulphur. However the control plants showed apparent signs of a nutrient deficiency, and only one sample was tested from each cultivar and treatment. Therefore the experiment was repeated with more vigorous plants which had been potted on more rapidly and that would yield enough material to give three samples. Plants were harvested at 13 dpi (initial symptoms in susceptible plants, no symptoms in intermediate resistant plants) and 22 dpi (severe symptoms in susceptible plants, mild symptoms on the lowest

leaves of intermediate resistant plants). In this experiment "low" levels of elemental sulphur were present in control vascular tissue at both time points. The vascular tissue from susceptible pathogen-inoculated plants contained a similar "low" amount of S_8 to controls at 13 dpi with 31.8 ± 3.08 ng/g. This was increased slightly at 22 dpi to 49.9 ± 16.12 ng/g. The vascular tissue from intermediate resistant *F. oxysporum*-inoculated plants again showed a more rapid and substantial accumulation in elemental sulphur reaching 140.5 ± 33.7 ng/g at 13 dpi and decreasing slightly at 22 dpi to 127.0 ± 9.6 ng/g (Fig. 3.16).

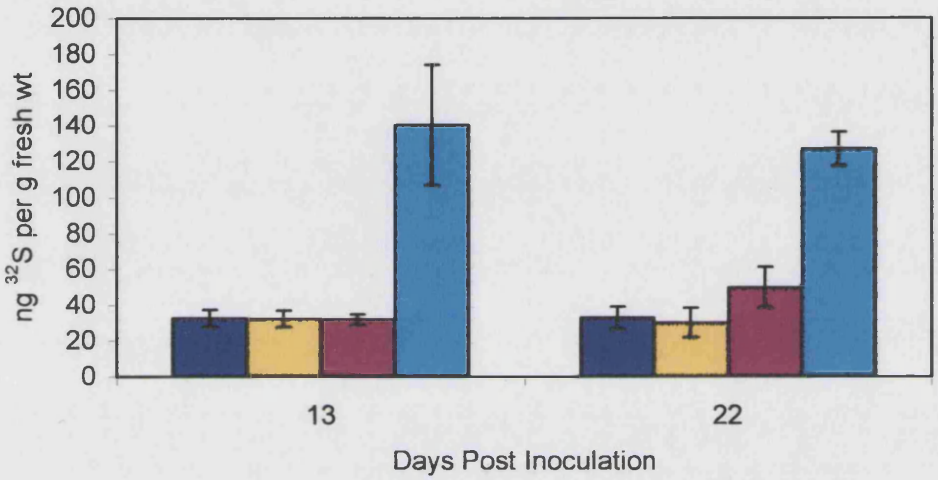

Figure 3.16 GC-MS analysis for S_8 of xylem tissue from intermediate resistant and susceptible French bean plants inoculated with *F. oxysporum* f. sp. *phaseoli*. Three pooled samples each of xylem tissue from five control (■) and pathogen-inoculated (■) susceptible plants, and control (■) and pathogen-inoculated (■) intermediate resistant plants were harvested at 13 dpi (initial symptoms in susceptible plants, no symptoms in intermediate resistant plants) and 22 dpi (severe symptoms in susceptible plants, mild symptoms on the lowest leaves of intermediate resistant plants) for extraction and analysis by GC-MS. Values represent the mean with SE.

3.3.2 Elemental sulphur analysis in plants inoculated with bacterial vascular pathogens

3.3.2.1 Tomato vs. *R. solanacearum*

Disease symptoms

Disease symptoms became apparent in susceptible (Super Marmande) tomato plants inoculated with *R. solanacearum* at around 7 dpi. At first plants showed signs of water stress in the lower leaves, which rapidly progressed into severe flaccidity of all leaves within 6h. By 14 dpi the whole plant including the stem was flaccid, the lower leaves chlorotic and the plant severely stunted in comparison to pathogen-inoculated intermediate resistant and healthy plants (Fig. 3.17).

Intermediate resistant (Hawai 7996) plants inoculated with *R. solanacearum* also showed slight chlorosis of lower leaves at 14 dpi but otherwise were indistinguishable from controls.

(a) (b)

Figure 3.17 Symptoms expressed by susceptible (Super Marmande) tomato plants inoculated with *R. solanacearum* at 14 dpi. Susceptible tomato plants root-inoculated with *R. solanacearum* (a) expressed severe symptoms of water stress, stunting and chlorosis in the lower leaves in comparison to water-inoculated control plants (b) at 13 dpi. Note, intermediate resistant (Hawai 7996) tomato plants challenged with *R. solanacearum* showed slight chlorosis of lower leaves at this time point but were otherwise indistinguishable from controls. This is the second time point at which xylem was analysed for elemental sulphur.

GC-MS analysis of S_8 in xylem from susceptible and intermediate resistant tomato lines

Plants were harvested at both 7 dpi (initial symptoms in susceptible plants, no symptoms in intermediate resistant plants) and 14 dpi (severe symptoms in susceptible plants and mild symptoms only on the lowest leaves of intermediate resistant plants). Control plants showed "low" levels of elemental sulphur in the xylem tissue. Susceptible pathogen-inoculated plants accumulated 71.1 ± 9.48 ng/g of elemental sulphur in the vascular tissue at 7 dpi which increased to 183 ±15.2 ng/g at 14 dpi. Again the response to the pathogen in the intermediate resistant cultivar was more rapid and substantial reaching 155 ± 16.1 ng/g at 7 dpi which increased to 213 ± 36.5 ng/g at 14 dpi. (Fig. 3.18).

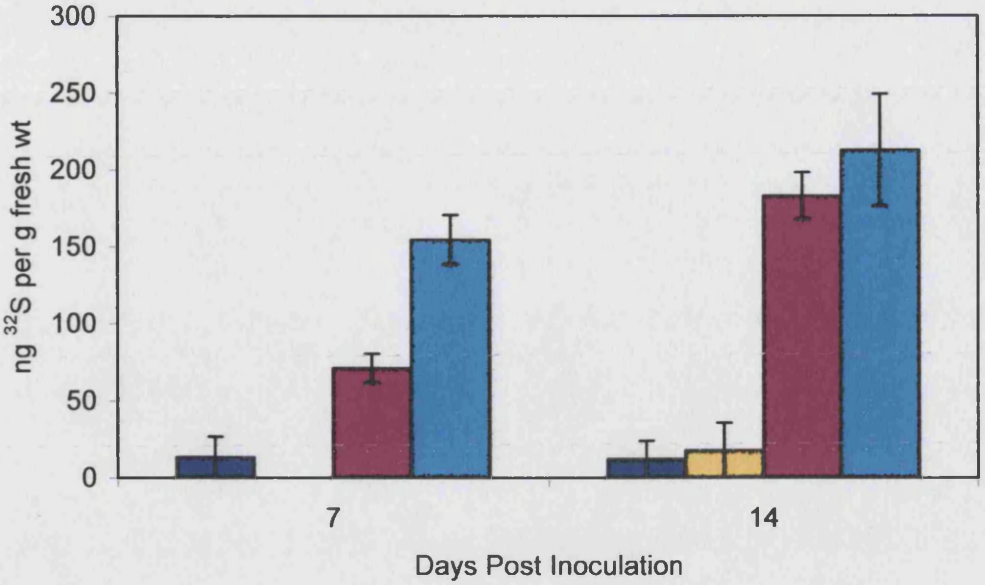

Figure 3.18 GC-MS analysis for S_8 of xylem tissue from intermediate resistant and susceptible tomato plants inoculated with *R. solanacearum*. Three pooled samples each of xylem from three control (■) and pathogen-inoculated (■) susceptible plants, and control (■) and pathogen-inoculated (■) intermediate resistant plants were harvested at 7 dpi (initial symptoms in susceptible plants, no symptoms in intermediate resistant plants) and 14 dpi (severe symptoms in susceptible plants, mild symptoms only in the lowest leaves of intermediate resistant plants) for extraction and analysis by GC-MS. Values represent the mean with SE.

3.3.2.3 Maize vs. *E. stewartii*

Disease symptoms

Symptoms became evident in susceptible sweet corn (Jubilee) leaves at around 8 dpi with water soaking of the tissue immediately surrounding the inoculation sites. In the following days water-soaked areas developed into elongating chlorotic lesions that by 14 dpi had spread a substantial distance along the leaf (Fig. 3.19(d)). In intermediate resistant field corn (IFSI 90-1) water soaking and subsequent lesions were limited to *ca.* 1 cm from the inoculation site (Fig. 3.19(b)). Control plants showed a small area of necrosis directly surrounding the wound site but no water soaking or chlorotic lesions (Figs. 3.19(a) and (c)).

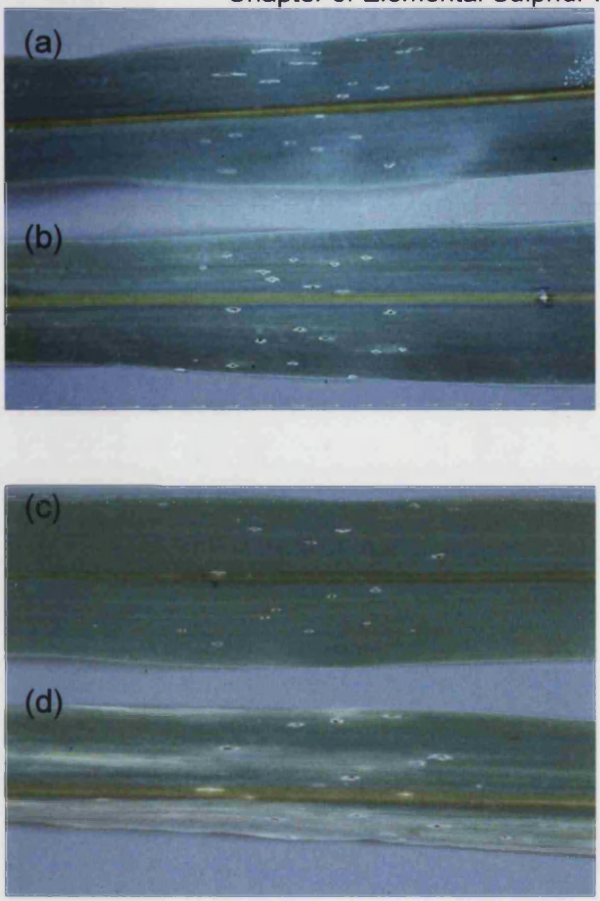

Figure 3.19 Symptoms expressed by susceptible (Jubilee) and intermediate resistant (IFSI 90-1) maize leaves inoculated with *E. stewartii* at 14 dpi. Leaves were inoculated with a clapper (Fig. 3.1) causing wounding of the leaves with pins and subsequent infection of the wounds by *E. stewartii*. In water-inoculated control leaves, only wounds made by the clapper could be detected at 14 dpi (a) and (c). In the intermediate resistant leaves, small lesions (*ca.* 1 cm) developed around the wounds (b) in which the pathogen was contained. In susceptible leaves elongating chlorotic lesions had developed along the length of the leaves from the inoculation site at this time point (d). These lesions eventually spread systemically throughout the plant. This is the time point at which leaves were harvested for elemental sulphur analysis.

GC-MS analysis of S_8 in xylem from susceptible and intermediate resistant maize lines

In an initial experiment on maize leaves harvested at 14 dpi (large spreading lesions on leaves of susceptible plants, small defined lesions on leaves of intermediate resistant plants) similar and "low" levels of elemental sulphur were detected in both control and pathogen-inoculated samples. The experiment was not repeated.

3.3.3 Elemental sulphur analysis in plants inoculated with bacterial leaf pathogens

3.3.3.1 Various plants vs. incompatible *P. syringae* pathovars

Development of a hypersensitive lesion

Symptoms became evident in all plants (barley, French bean, tomato, lettuce, cabbage, tobacco and *Arabidopsis*) inoculated with *P. syringae* by 24h post-inoculation with collapse and desiccation of infected host tissues. This was followed by the rapid appearance of a restricted lesion clearly delimited from the surrounding healthy tissue (Fig. 3.20).

(a) (b) (c)

Figure 3.20 Leaves responding hypersensitively to challenges with an incompatible *P. syringae* pathovar. Leaves of various plants including tomato (a), lettuce (b) and *Arabidopsis* (c) were infiltrated with an incompatible *P. syringae* pathovar to produce hypersensitive lesions all over the leaf. These are the lesions at 5 dpi. Leaves were harvested at this time point and at various earlier time points during lesion development for elemental sulphur analysis.

GC-MS analysis of S_8 in leaves responding hypersensitively to *P. syringae*

Initially all plant species except *Arabidopsis* were harvested for elemental sulphur analysis at 7 dpi and in later experiments plants were harvested at 1, 3 and 5 dpi. All pathogen-inoculated samples including those extracted from whole leaves and from excised hypersensitive lesions contained similar and "low" amounts of elemental sulphur as did water-inoculated and uninoculated controls.

Arabidopsis leaves were harvested initially at 4 dpi (established hypersensitive lesions on challenged leaves) and then at 1 dpi (early lesion development) and 5 dpi (established hypersensitive lesions) in later experiments. All leaves, whether uninoculated, inoculated with water or *P. syringae,* in contrast to other species were found to contain a constitutive amount of elemental sulphur at both time

points. In the initial experiment the uninoculated and water-inoculated control leaves contained 6.46 and 6.13 μg/g S_8 respectively whereas the amount found in *P. syringae*-challenged leaves was 0.33 μg/g. However only one sample per treatment was tested and so the experiment was repeated to give three samples per treatment. Uninoculated and water-inoculated leaves again contained a constitutive amount of elemental sulphur at both 1 and 5 dpi but lower levels were present compared to the previous experiment ranging from 1015.2 ± 87.8 to 1564.4 ± 626.8 ng/g across both time points. In this experiment *P. syringae*-inoculated leaves also contained a similar level of elemental sulphur with 1167.5 ± 283.3 ng/g at 1 dpi and 1733 ± 309.7 ng/g at 5 dpi (Fig. 3.21).

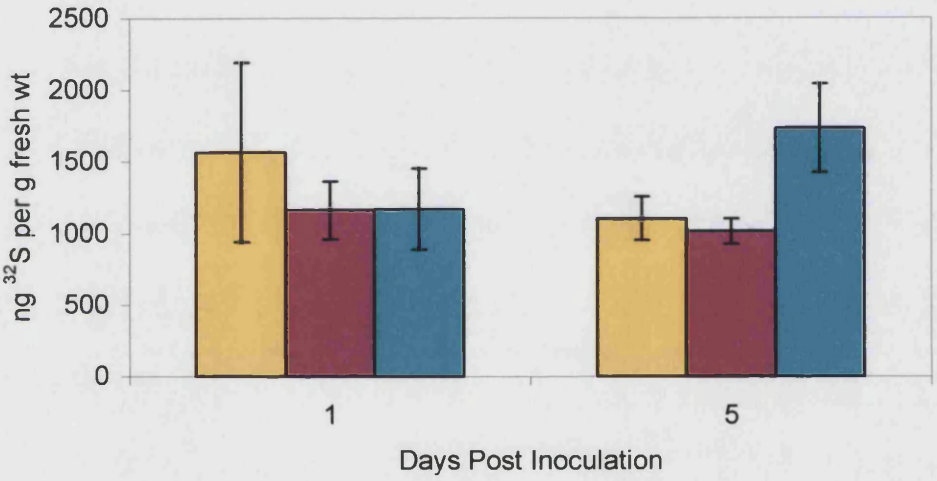

Figure 3.21 GC-MS analysis for S_8 in *Arabidopsis* leaves. Leaves were either uninoculated (■), water-inoculated (■) or *P. syringae*-inoculated (■) and at 1 dpi (early lesion development on challenged leaves) and 5 dpi (established hypersensitive lesions) three pooled samples were harvested from 24 plants from each treatment for GC-MS analysis. Values represent the mean with SE.

Attempted localisation of sulphur in tobacco leaves showing a HR to *P. syringae*

P. syringae-inoculated hypersensitive necrotic lesions with a 3 mm non-necrotic border and water-inoculated control leaf areas were excised from tobacco plants at 7 dpi. Lesions were cryofixed, fractured transversely (in such a way that both necrotic hypersensitive cells and those living cells immediately around the lesion could be analysed), aluminium coated and viewed by SEM. General area X-ray analyses (*ca.* 10 per fracture) were carried out along each fracture focusing particularly at the cells at the lesion border but also on the cells either side of the border for the detection of sulphur. Spot analyses (*ca.* 10 per fracture) were also

carried out on individual necrotic and healthy cells. Only very low levels of sulphur were detected in comparison to the levels of both phosphorus and the predominant endogenous element potassium in all areas and cells analysed from both water-inoculated and *P. syringae*-inoculated tissues.

Attempted localisation of sulphur in uninoculated *Arabidopsis* leaves

Uninoculated *Arabidopsis* leaves were cryofixed, fractured transversely and aluminium coated prior to viewing by SEM. Again approximately 10 general area X-ray analyses each encompassing many cells were made across the leaf as well as approximately ten spot analyses on individual cells including vascular cells, bundle sheath cells, epidermal cells and cuticle, spongy mesophyll cells and palisade cells. All areas and cell types contained an equally "high" level of sulphur (≥ 50% and occasionally greater than of the height of the potassium peak) (Fig. 3.22).

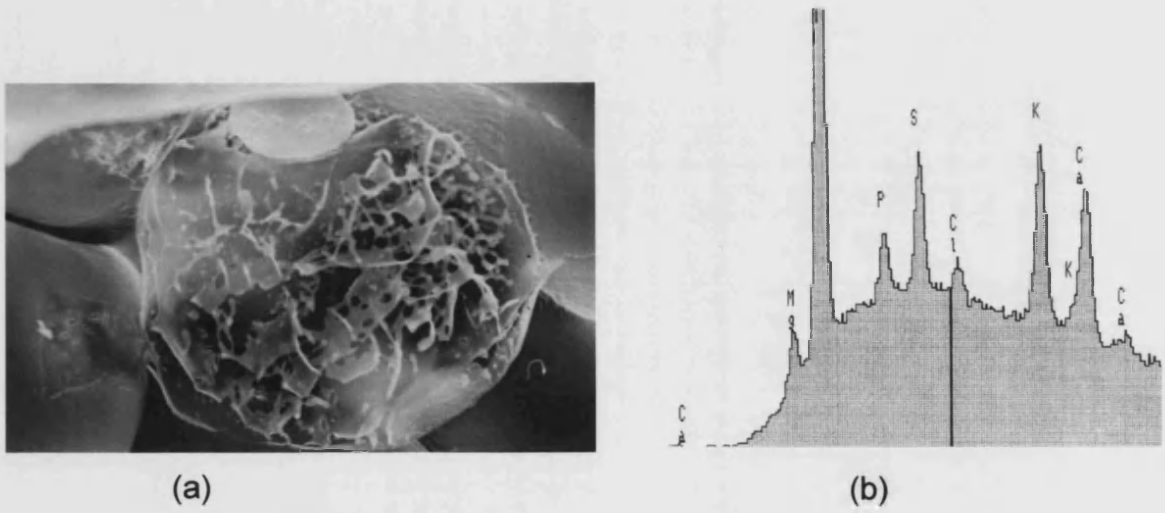

Figure 3.22 Sulphur detected by an X-ray spot analysis on a mesophyll cell of an uninoculated *Arabidopsis* leaf. *Arabidopsis* leaves were cryofixed, fractured transversely and viewed by SEM. General areas of cells and individual cells were analysed by X-ray analyses. The image (a) is of a fractured *Arabidopsis* mesophyll cell and the spectrum (b) was produced by an X-ray spot analysis on that cell. A "high" (>50% of K peak) level of sulphur similar to that found in the mesophyll cell occurred in all cell types within the leaves.

3.3.4 Elemental sulphur analysis in plants inoculated with fungal leaf pathogens

3.3.4.1 *B. oleracea* vs. *Peronospora parasitica*

Development of hypersensitive lesions (assessed by J. Byrne)

Symptoms became visible on *B. oleracea* cotyledons at around 3 dpi and were similar to those previously described by the infiltration of incompatible *P. syringae* to leaves. Restricted lesions were evident as very small flecks of necrosis clearly delimited from the surrounding healthy tissue at 4 to 5 dpi (Fig. 3.23).

(a) (b)

Figure 3.23 *B. oleracea* cotyledons challenged with *P. parasitica*. Cotyledon (a) has responded hypersensitively to a challenge with an incompatible accession of *P. parasitica* and restricted lesions are evident at 5 dpi as very small flecks of necrosis clearly delimited from the surrounding healthy tissue. In comparison cotyledon (b) is showing susceptible symptoms typical of this downy mildew after infection with a compatible accession of *P. parasitica* at this time point. On the underside of the cotyledon profuse downy growth of greenish-white sporangiophores have developed.

GC-MS analysis of S_8 in *B. oleracea* cotyledons responding hypersensitively to *P. parasitica*

In an initial experiment on *B. oleracea* cotyledons harvested at 4 dpi (established hypersensitive lesions on challenged leaves) 372 ng/g of elemental sulphur was detected in control leaves and 460 ng/g was detected in *P. parasitica*-challenged leaves. Only one pooled sample of each treatment was tested and no more material was provided.

3.3.5 Elemental sulphur analysis of *V. dahliae*

GC-MS analysis of S_8 in *V. dahliae* spores, mycelium and culture fluids

To determine whether vascular invading fungi could produce elemental sulphur, *V. dahliae* spores, mycelium and culture fluids from 3d shake cultures grown in Czapek dox liquid medium were analysed. They contained only very "low" levels of elemental sulphur. As previously explained this meant that the peaks corresponding to elemental sulphur were no higher than surrounding background peaks on the GC-MS spectrum and so were unlikely to actually be elemental sulphur (P. Gaskin pers. comm.). Even if they did correspond to elemental sulphur they would only represent 0.1 – 0.15 ng S_8 per mL of culture.

3.4 Discussion

To date there are few examples of elemental sulphur production by eukaryotes. The discovery of elemental sulphur in resistant lines of *T. cacao* (Sterculiaceae), in sufficient quantities, in the right place, and at the right time potentially to inhibit *V. dahliae* was the first to implicate the element in induced disease resistance (Cooper et al., 1996; Resende et al., 1996).

It seems that elemental sulphur as an induced defence mechanism is far more widespread in higher plants as suggested here by its production in tomato (Solanaceae) and cotton (Malvaceae) in response to *V. dahliae*, tobacco (Solanaceae) and French bean (Leguminosae) in response to *F. oxysporum,* and tomato in response to *R. solanacearum.* For all of these interactions the kinetics and levels of elemental sulphur accumulation were revealed for the first time as a result of the development of a method for accurate quantification of $^{32}S_8$ (chapter 2). In all of the above interactions the pattern of elemental sulphur accumulation was found to resemble that of various other organic phytoalexins, with a more rapid and intensive production in the resistant or intermediate resistant varieties than in the susceptible genotypes. In contrast to the interaction between cocoa and *V. dahliae,* accumulation of elemental sulphur also occurred in all of the susceptible interactions. In tobacco and French bean elemental sulphur eventually increased to higher levels than in the challenged resistant plants, but this was associated with intensive colonisation by the pathogen and severe symptom development. Analogous patterns of phytoalexin accumulation in incompatible host-pathogen interactions include the isoflavonoids phaseollin in French bean (Bailey and Deverall, 1971) and glyceollin in soybean (Yoshikawa et al., 1978; Yoshikawa and Masago, 1982), the sesquiterpene rishitin in tomato (McCance and Drysdale, 1975; Gentile and Matta, 1976) and the furanoacetylene wyerone in broad bean (Hargreaves et al., 1977). Related plant families generally make use of chemically related compounds for defence. Elemental sulphur is perhaps the only known phytoalexin that is produced by so many different taxa, but this may reflect that it is the only known inorganic antimicrobial agent produced by plants (Dixon, 2001).

No elemental sulphur was detected in the compatible or incompatible interactions between strawberry (Rosaceae) and *V. dahliae*. The multigenic resistance of strawberry to *V. dahliae* is not that effective even in the most resistant varieties

and varies greatly, especially with the level of *V. dahliae* present in the soil (Harris and Yang, 1996; Shaw et al., 1997). The intermediate resistance of the cultivar Redgauntlet used in this survey was overcome by the pathogen but it took longer than for the susceptible cultivar. Furthermore, in a recent survey on the Rosaceae by Kokubun and Harborne (1994) phytoalexin production was only detected in about 15% of the species tested. In strawberry no phytoalexins were found in the woody tissue, leaves or fruits but several constitutive antifungal metabolites were detected. These constitutive compounds may substitute for an inability of strawberry plants to produce phytoalexins. Similarly no elemental sulphur was detected in the compatible and incompatible interactions between maize (Gramineae) and *E. stewartii*. Consideration of the literature on dicotyledonous plants reveals that fewer studies have focused on phytoalexins produced by monocotyledonous plants. Phytoalexins are produced by many members of the Gramineae and include the anthranilic acid avenalumins from oats, the stilbene piceatannol and anthocyanidin luteolinidin from sugar cane, the flavone sakuranetin from rice and the anthocyanidin luteolinidin from sorghum. No compound as yet, including elemental sulphur, has been implicated as a phytoalexin from maize (Harborne and Williams, 1994). However, whole leaves were harvested for elemental sulphur analysis of maize leaves instead of extracted xylem tissue as in tomato and tobacco. Therefore it is possible that if elemental sulphur were produced in the xylem of maize, the surrounding tissue may have diluted it.

In an initial experiment on French bean plants challenged with *F. oxysporum*, no accumulation of elemental sulphur was detected in either resistant or susceptible xylem in response to the pathogen, but sulphur was evident from a second experiment. In the initial experiment plants may have been slightly nutrient deficient, perhaps in sulphur; such deficiencies can be difficult to differentiate as many produce similar symptoms (Scaife et al., 1983). Work by S. Hall (unpublished data) suggested that high levels of supplied sulphate were important in the changes in sulphur metabolism induced during the interaction between resistant tomato plants and *V. dahliae*. Only those plants given a high sulphur nutritional regime showed significant increases in sulphate, glutathione and cysteine levels in the xylem tissue in response to the pathogen. This raises important questions as to how the nutrient status of the plant affects its ability to defend itself against pathogens and is discussed further in chapter 6.

Phytoalexin accumulation, often in combination with other defence-related factors, has long been implicated in the restriction of pathogen growth within cells undergoing a HR to incompatible pathogens (chapter 1). Cells adjacent to the hypersensitively responding cells are thought to produce phytoalexins that then accumulate within the hypersensitive lesion, thereby creating a toxic environment for the pathogen (Bailey, 1982). However analysis of tomato, tobacco, French bean, barley (Gramineae), lettuce (Compositae), cabbage and *Arabidopsis* (Brassicaceae) leaves undergoing hypersensitivity to incompatible *P. syringae* pathovars revealed no accumulation of elemental sulphur either by GC-MS or SEM-EDX in response to the pathogen. It seems that elemental sulphur production may not be induced as part of a general HR in all tissues, at least not in response to incompatible bacterial pathogens. This response may be specific to vascular tissue, in particular XP cells. Xylem-specific expression of genes is already known to occur. For example *pox A* and *pox N* peroxidase genes isolated from rice, up-regulated in response to wounding, were xylem-specific (Ito et al., 2000), and a cellulose synthase gene from aspen was specifically expressed in xylem tissue during both normal plant growth and mechanical stress (Wu et al., 2000). More importantly, group 2 sulphate transporters thought to be responsible for sulphate distribution within plants (chapter 4) (Smith et al., 1995a; Takahashi et al., 1997; Takahashi et al., 2000), and the dominant *I-2* gene required for the resistance of tomato to the fungal vascular pathogen *F. oxysporum* f. sp. *lycopersici* race 2 were expressed predominantly in the vascular tissue (Mes et al., 2000). Perhaps the *Ve* gene for the resistance of tomato to *V. dahliae* also is specifically expressed in the xylem tissue, initiating elemental sulphur production.

It is possible that elemental sulphur production may be induced in leaves of plants as part of a HR to incompatible fungal pathogens but this was not investigated thoroughly in the survey. Whether elemental sulphur can be induced in leaf tissue by an incompatible fungus will be investigated subsequently using the interaction between *Cladosporium fulvum* and tomato. The incompatible interaction between tomato and *V. dahliae* yielded much more elemental sulphur per g of xylem than the incompatible interaction between tomato and *R. solanacearum* (10.4 ± 1.7 μg/g maximum sulphur accumulated in comparison to 0.21 ± 0.04 μg/g) which might suggest that fungal elicitors are more efficient at inducing elemental sulphur production than bacterial elicitors. Early surveys also indicated that bacteria caused less phytoalexin accumulation than fungi; for example several bacteria

failed to cause pisatin to form in pea pods and less phaseollin was produced by pods of French bean in response to bacteria than to fungi (Cruickshank and Perrin, 1963; Cruickshank and Perrin, 1971). However this is not true in all cases as many bacteria are also good elicitors of phytoalexin production. Examples include the production of high concentrations of the fluorescent sesquiterpene phytoalexins by cotton in response avirulent strains of *Xanthomonas campestris* pv. *malvacearum* (Essenberg et al., 1992; Pierce et al., 1996) and large concentrations of phaseollin were found in French bean leaves that had responded hypersensitively to *P. syringae* pv. *phaseolicola* (Stholasuta et al., 1971). An alternative explanation for the differences in elemental sulphur production in tomato xylem between the interaction with *V. dahliae* and *R. solanacearum* is related to the genetic basis for resistance. It is possible that the monogenic resistance of tomato to *V. dahliae* and the multigenic resistance of tomato to *R. solanacearum* require different signalling events that eventually both induce production of elemental sulphur but more or less intensively. To date there is limited knowledge on the mechanisms of multigenic resistance but it is thought that many of the genes are directly involved in the expression of defence responses, unlike most genes involved in monogenic resistance that appear to code for pathogen-recognition. Thus there may be differences in the activation and expression of defence mechanisms between the two systems (Heath, 2000b).

Elemental sulphur accumulation in response to an incompatible fungal pathogen was investigated in *B. oleracea* (cabbage/senna hybrid) (Brassicaceae) cotyledons challenged with *P. parasitica*. However surprisingly, both pathogen-inoculated and water-inoculated cotyledons were found to contain significant constitutive levels of elemental sulphur. Very high levels of constitutive elemental sulphur were also detected in uninoculated *Arabidopsis* leaves, and SEM-EDX revealed that all cell types within these leaves contained sulphur. The production of constitutive elemental sulphur may therefore be a property of the Brassicaceae family, but not of all members, as none was detected in the leaves of *B. oleracea* (cabbage). The amount of elemental sulphur produced even within a species may vary quite considerably. Considering data from *Arabidopsis,* the elemental sulphur content of leaves varied from 1 to 6 μg/g between experiments and so may accumulate more or less intensively under certain, as yet undefined, conditions. The phenomenon of constitutive elemental sulphur in plants has previously only been described in the cuticular waxes of various *Pinus* spp., *Picea abies*, various *Yucca* spp., *Nerium*

oleander, Phragmites communis, Tilia cordata and *Geranium sylvaticum* none of which are members of the Brassicaceae (Kylin et al., 1994). Whether the elemental sulphur produced in the leaves of *Brassica* plants is synthesised in the same way as that which accumulates in waxes or that which is produced by xylem tissue in response to vascular pathogens is not known. As yet there is no biosynthetic pathway for elemental sulphur characterised in eukaryotes.

Coincidentally, *Brassica* plants are known to be sulphur-rich plants. The phytoalexins that they produce all contain at least one sulphur atom (Pedras et al., 1997) and they possess the sulphur-containing glucosinolate phytoanticipins (chapter 1). SEM-EDX does not differentiate between elemental sulphur and bound sulphur. This may explain why all cells within the *Arabidopsis* leaf transverse section were shown to contain high levels of sulphur, and so the precise localisation of elemental sulphur within *Arabidopsis* leaves will be difficult to assess by this method. It is possible that elemental sulphur may be produced by the breakdown of glucosinolates which, in the presence of myrosinases, form an unstable intermediate, that at pH 2 to 5 can spontaneously rearrange to produce nitriles and elemental sulphur (Bones and Rossiter, 1996; Foo et al,. 2000). Elemental sulphur production by this process has been largely ignored in the literature on glucosinolate breakdown. Glucosinolates are only thought to be degraded by myrosinase on mechanical damage of the plant tissue. If elemental sulphur is produced constitutively by glucosinolate breakdown then there must be either some leakage of the system or a process that allows their degradation without plant wound responses. No such phenomenon has been described as yet and such a study would require knowledge of the precise localisation of myrosinases, glucosinolates and elemental sulphur, of which the latter two are unknown (Bones and Rossiter, 1996). Why cabbage leaves did not contain constitutive amounts of elemental sulphur is uncertain, but may relate to the presence of different glucosinolates (over 120 different glucosinolates are now known to exist) to *Arabidopsis* or the cabbage/senna hybrid (Fahey et al., 2001), or different cellular conditions that result in different glucosinolate breakdown products (Chen and Andreasson, 2001), or different conditions for plant growth. Furthermore, the age of the leaves/cotyledons analysed may have an effect. For cabbage, older leaves were harvested at 49 to 56 d after planting whereas the cabbage/senna hybrid cotyledons were harvested at 13d after planting. In oil seed

rape it was found that in older leaves the glucosinolate content was much lower than in younger leaves (Porter et al., 1991).

Although *T. cacao* is claimed to produce glucosinolates (Gill et al., 1984), tomato, tobacco, cotton, and French bean do not and so the elemental sulphur induced in xylem tissue by these plants in response to vascular pathogens probably originates from a different biochemical pathway that is as yet uncharacterised. Of all the incompatible vascular interactions that resulted in elemental sulphur production, the interaction between tomato and *V. dahliae* offers the most tractable model for genetical and biochemical investigation of origin, as initiated in chapter 4. Inoculation of near isogenic resistant and susceptible tomato lines resulted in rapid, acropetal, systemic spread of *V. dahliae* in susceptible plants whereas colonisation of the resistant variety was restricted mainly to the roots (0.3% of vessels infected only at the first internode). One out of three plants gave a positive reisolation at internodes 1 and 8 at 28 dpi but no quantitative reisolation. This can be accounted for by the occasional spores being carried a long distance up the plant due to imperfect vessel endings (Presley and Taylor, 1969; Beckman et al., 1976). This expression of the *Ve* gene for resistance concurs with previous data of Cooper and Wood (1980). The overall restriction and eventual disappearance of the hyphae from the stem base in resistant plants implies the production of antifungal compounds. Elemental sulphur may contribute to this antimicrobial environment. Its presence in tomato xylem where fungal colonisation was absent confirms that the elemental sulphur is of plant and not fungal origin. Furthermore, analysis of fungal spores, mycelium and culture fluids of *V. dahliae* grown in Czapek dox, a basal medium that is intended to simulate growth in xylem sap (Pegg, 1981), also revealed no elemental sulphur production by the fungus.

The antimicrobial environment within the plant is thought to be maintained by physical occlusion of vessels with tyloses and gels (Hutson and Smith, 1980). In this study, occlusions were abundant in the incompatible tomato vs. *V. dahliae* interaction but were significantly less in the infected xylem of the susceptible genotype. The kinetics and levels of elemental sulphur accumulation revealed by GC-MS of a more rapid and intensive production in the resistant than susceptible host were also suggested by SEM-EDX. This differential response was especially remarkable as elemental sulphur accumulation was inversely related to the

amount of pathogen; fungal biomass was negligible in contrast to the extensive colonisation of the xylem in the susceptible cultivar.

SEM-EDX revealed that accumulation of sulphur as the element and/or as organically bound sulphur was widespread in tomato vascular tissue undergoing an incompatible interaction with *V. dahliae*. Occasionally, intense localisations were detected in scattered XP cells, vascular gels, tyloses and in xylem vessel walls in pathogen-inoculated plants and they were detected in both cryofixed and lyophilised sections. These results corresponded closely with the results of SEM-EDX on vascular tissues from resistant *T. cacao* plants inoculated with *V. dahliae* (Cooper et al., 1996). Terpenoid aldehyde phytoalexins of cotton are similarly formed in XP cells and these along with phytoalexins of some other species are exuded into xylem to impregnate vascular occlusions (Bell and Mace, 1981). Impregnation of these structures with elemental sulphur would be of direct relevance to resistance against vascular fungi by providing an effective barrier to vertical and lateral spread, which characterises their mode of invasion (Beckman and Roberts, 1995; Cooper, 2000).

The amount of elemental sulphur detected in entire tissue extracts in relation to toxicity to the challenging pathogens is discussed in more detail in chapter 5. However, it is important to note here that calculations of the amounts of other phytoalexins such as phaseollin, have revealed that whole tissue extracts give a gross underestimate of the actual amounts present, because phytoalexins become concentrated in hypersensitive cells where they can be in considerable excess above that required for pathogen inhibition (Bailey and Deverall, 1971). SEM-EDX analysis has revealed that elemental sulphur may also be concentrated in this way.

In conclusion it appears that the ability of plants to produce elemental sulphur is not limited to the cocoa plant and is more widespread within the plant kingdom. Elemental sulphur has been shown here to accumulate in the vascular tissues of plants from several families (Solanaceae, Malvaceae, Leguminosae) in response to both bacterial and fungal vascular pathogens. This phenomenon seems to be specific to the xylem tissue as elemental sulphur never accumulated in leaf tissue of plants from many families (Solanaceae, Leguminosae, Brassicaceae, Compositae) in response to avirulent pathogens. Furthermore not all plants appear

capable of producing elemental sulphur with strawberry (Rosaceae) and maize (Gramineae) showing no accumulation in response to vascular pathogens, although some members of the Brassicaceae appear to contain constitutive elemental sulphur.

As previously described (chapter 1), to implicate a phytoalexin in resistance it must be present in the right place, in sufficient quantities and at the right time to inhibit the pathogen (Osbourn, 1999). All of these criteria were fulfilled when implicating elemental sulphur accumulation in the defence of cocoa to *V. dahliae.* Elemental sulphur accumulation in the vascular tissues of all elemental sulphur-producing plants analysed here occurred more rapidly and intensively in the resistant varieties than in the susceptible genotypes in response to the vascular pathogens. This suggests that elemental sulphur would be present at the right time to play a role in defence in all of these cases. For the tomato vs. *V. dahliae* interaction sulphur also accumulated in structures of direct relevance to defence against xylem invading pathogens. To fulfil all of the criteria of Osbourn (1999) for the tomato vs. *V. dahliae* interaction it now remains to be shown that elemental sulphur is produced in sufficient quantities in tomato xylem potentially to inhibit *V. dahliae in vivo* (chapter 5).

Chapter 4

Elucidation of the Biosynthesis of Elemental Sulphur in Tomato by Investigating Gene Expression

4.1 Introduction

Elemental sulphur production in eukaryotes is by an uncharacterised biosynthetic pathway(s). It may begin from one or a number of precursors and involve known or novel genes and enzymes. Before formulating a model for elemental sulphur biosynthesis in plants it is important to be aware of normal sulphur assimilation pathways including the intermediates, genes and enzymes involved. However, sulphur metabolism in plants has been far less well studied than nitrogen metabolism and some areas of the assimilation pathway have only recently been resolved (Leustek et al., 2000). For a summary of the leading hypotheses for sulphur assimilation in plants see Fig. 4.1.

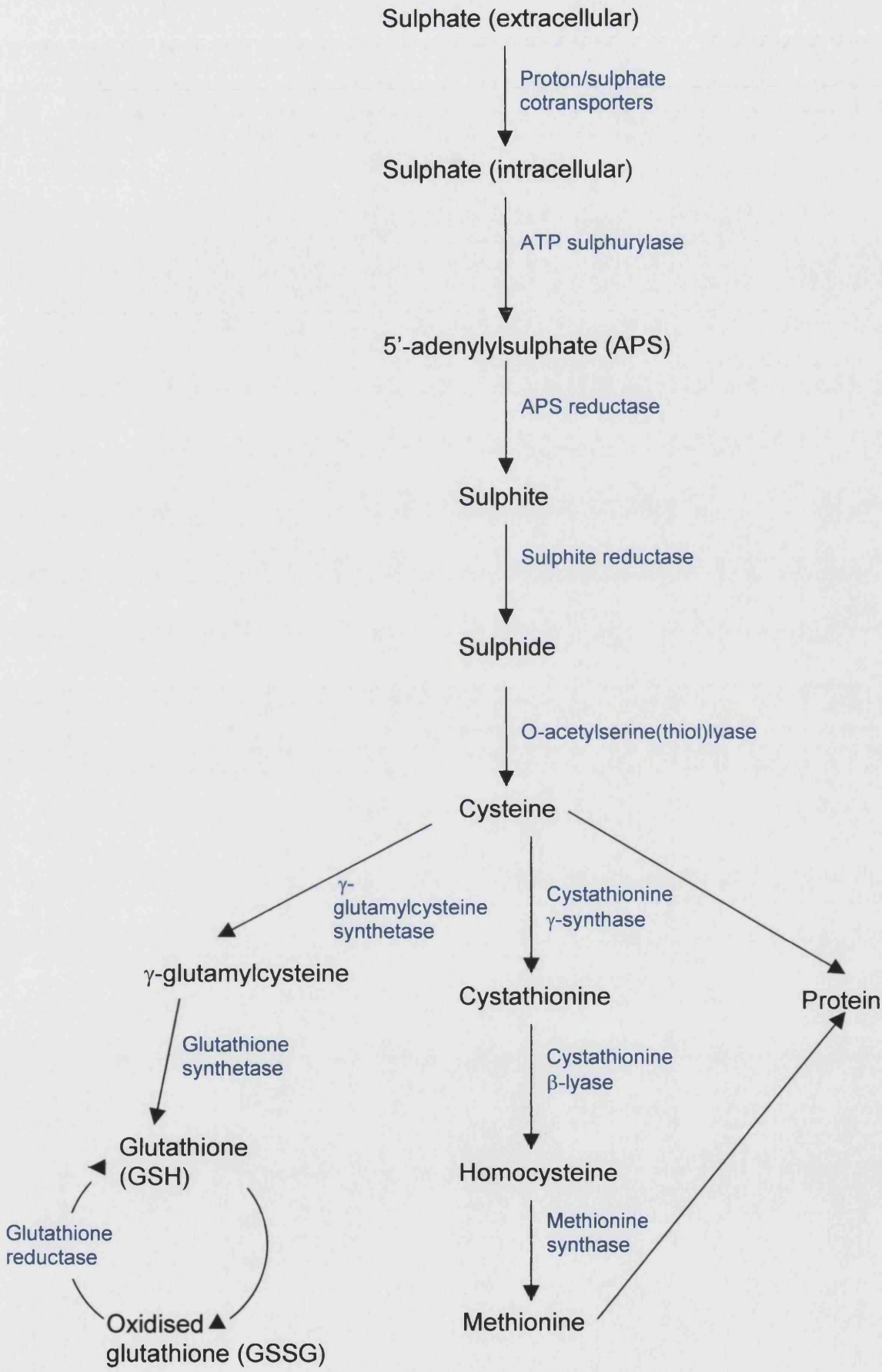

Figure 4.1 Sulphur assimilation in higher plants. The intermediates and products of sulphur assimilation are shown in black and the enzymes involved in their production in blue. All of the enzymes involved have been cloned in higher plants. For regulation of this pathway please refer to the text.

The sulphate anion serves as the primary sulphur source for plants. It is actively transported into the roots from the soil where it can remain or be transported throughout the plant (Leustek et al., 2000). Transport into cells across the plasma membrane is mediated by proton/sulphate co-transporters that are driven by a proton gradient generated by ATPases (Clarkson et al., 1993). These transporters have been found in many plant species including the tropical legume *Stylosanthes hamata*, *Arabidopsis*, soybean, barley, maize, *Brassica juncea* (Indian mustard), resurrection grass and tomato. In most species they appear to be encoded by a gene family (Kouchi and Hata, 1993; Smith et al., 1995a; Ng et al., 1996; Takahashi et al., 1996; Smith et al., 1997; Takahashi et al., 1997; Yamaguchi et al., 1997; Bolchi et al., 1999; Takahashi et al., 1999; Vidmar et al., 1999; Takahashi et al., 2000; Vidmar et al., 2000; J. Howarth and M. Hawkesford, IACR Rothamsted, Harpenden, UK, unpublished data). Sulphate transporters have been classified into four groups according to the similarities of their protein sequences, kinetic properties and tissue specific localisations, and each group is thought to perform a specialised function within the plant as regards sulphur uptake and distribution (Grossman and Takahasi, 2001; Yoshomoto et al., 2002). Group 1 transporters are primarily expressed in the roots of plants under conditions of sulphur starvation and these are thought to enhance sulphur uptake from the soil to compensate for the sulphur deficiency. Group 2 transporters have a lower affinity for sulphate and are expressed only in the vascular tissues, implicating them in internal distribution of sulphate within the plant. These group 1 and 2 transporters are the most common sulphate transporters found so far in higher plants and are also the best characterised (Smith et al., 1995a; Smith et al., 1997; Takahashi et al., 1997; Bolchi et al., 1999; Vidmar et al., 1999; Takahashi et al., 2000; Vidmar et al., 2000). Group 3 transporters share significant sequence similarities and are expressed preferentially in leaves although their role has not yet been characterised (Yoshimoto et al., 2002). Once within cells, sulphate can be stored within the vacuole or it can be metabolised. The sulphate reduction part of sulphur metabolism occurs within the plastids and so there must be some kind of sulphate transporter that allows sulphate into the plastid. The nature of this transporter is the subject of much debate and has not as yet been conclusively identified (Clarkson et al., 1993; Leustek et al., 2000). One possibility is a sulphate transporter with an amino terminal sequence that resembles a plastid transit peptide that has been found in *Arabidopsis*. This plastid sulphate transporter has been named a group 4 transporter and has similarities with

putative sulphate transporter genes in algae (Takahashi et al., 1999; Yoshimoto et al., 2002). So far in tomato, two distinct group 1 (LeST1 and LeST2) sulphate transporters have been cloned (J. Howarth and M. Hawkesford, unpublished data).

Once inside the plastid, sulphate metabolism begins with an adenylation reaction to activate the sulphate. This is catalysed by ATP sulphurylase and forms 5'-adenylylsulphate (APS) (adenosine 5'-phosphosulphate) (Leustek et al., 2000). Although this enzyme is mainly located in plastids there is also a minor cytosolic form (Lunn et al., 1990; Renosto et al., 1993; Hatzfeld et al., 2000). ATP sulphurylase has been found in several plants including spinach, potato, *B. juncea* and *Arabidopsis* and is encoded by a gene family that gives rise to different isoenzymes in each plant (Renosto et al., 1993; Klonus et al., 1994; Murillo and Leustek, 1995; Heiss et al., 1999; Hatzfeld et al., 2000). Although no ATP sulphurylase genes have as yet been cloned from tomato, two have been cloned in the closely related potato (Klonus et al., 1994). As well as catalysing the adenylation of sulphate, ATP sulphurylase may also play a role in regulating sulphur assimilation, because in general levels of mRNA increase slightly (≤ two fold) with sulphur starvation and decrease slightly (≤ two fold) when sulphur is supplied (Logan et al., 1996; Lappartient et al., 1999). However experiments using transgenic plants that overexpress ATP sulphurylase have given contrasting results as to whether this increases rates of sulphur assimilation (Hatzfield et al., 1998; Pilon-Smits et al., 1999).

Once sulphate is activated it is then reduced to sulphide, again in the plastids (Brunold and Suter, 1989; Leustek et al., 2000). The exact mechanism of how this occurs has been the subject of much debate and is clearly complex. Information on all of the possible pathways of sulphate assimilation is described by Hell (1997) but the leading hypothesis for sulphide production from sulphate in plants is described below. The pathway begins with APS reductase, which converts the sulphur in APS to sulphite using electrons derived from reduced glutathione (GSH) (Bick et al., 1998; Bick and Leustek, 1998). APS reductase genes have now been cloned from a number of plant species including *Arabidopsis, B. juncea, Catharanthus roseus* (Madagascar periwinkle), *Lemna minor* (common duckweed) and potato (Gutierrez-Marcos et al., 1996; Heiss et al., 1999; Prior et al., 1999; Suter et al., 2000; L. Hopkins and M. Hawkesford, IACR Rothamsted, Harpenden, UK, unpublished data). APS reductase is thought to be a key regulator of sulphate

reduction as mRNA levels increase far more than that of ATP sulphurylase in response to either sulphate starvation or conditions that increase demand for the products of sulphate assimilation (cysteine and glutathione) (Gutierrez-Marcos et al., 1996; Takahashi et al., 1997; Heiss et al., 1999; Leustek et al., 2000). Following reduction to sulphite, sulphite reductase is thought to catalyse the formation of sulphide using electrons from reduced ferredoxin (Leustek et al., 2000). This enzyme has been purified and the gene cloned only for *Arabidopsis* and maize (Bork et al., 1998; Nakayama et al., 2000). Sulphite reductase gene expression is not considered to be involved in regulation of sulphate assimilation (Bork et al., 1998; Leustek et al., 2000).

The incorporation of sulphide into cysteine is the last step in the reduction of sulphate. This reaction is catalysed by *O*-acetylserine(thiol)lyase (OASTL) using *O*-acetylserine (OAS) (Leustek et al., 2000). OAS is synthesised by serine acetyltransferase (SAT) from the substrates serine and acetylCoA. OASTL and SAT can associate through a protein-protein interaction into a bi-enzyme complex, originally called the cysteine synthase complex (Bogdanova and Hell, 1997). However free OASTL synthesises cysteine more efficiently than the complex, and furthermore, complex formation alters the kinetics of SAT from Michaelis-Menton type to positive co-operativity (a form of allosteric regulation in which the kinetics of a bisubstrate enzyme is highly sensitive to small changes in substrate concentration) with respect to its substrates (Droux et al., 1998). This indicates that OASTL is the true cysteine synthase and furthermore it suggests that OASTL may be a regulatory subunit that regulates SAT in response to OAS and sulphide. Complex formation is promoted by sulphide and complex dissociation promoted by OAS (Droux et al., 1998). It is thought that if the concentration of OAS increases due to insufficient sulphide production during sulphur starvation (Kim et al., 1999) then disruption of the complex would inhibit production of OAS. However, if the production of OAS was not rapid enough, then sulphide would accumulate and complex formation would be favoured to stimulate production of OAS (Leustek et al., 2000). This system would require some kind of regulation at the level of sulphate reduction and OAS and cysteine may play a role in this by regulating sulphate transporters and other enzymes involved in sulphate reduction (Herschbach and Rennenberg, 1991; Neuenschwander et al., 1991; Herschbach and Rennenberg, 1994; Lappartient and Touraine, 1996; Smith et al., 1997; Bolchi et al., 1999). OASTL and SAT are the only sulphur assimilation enzymes localised

in the plastids, cytosol and mitochondria. Only the plastids have all the enzymes needed for complete sulphate reduction and cysteine formation (Lunn et al., 1990; Rolland et al., 1992; Ruffet et al., 1994). It may be that cysteine cannot be exported from plastids but sulphide is able to diffuse out, thereby allowing cysteine to be produced elsewhere. Cysteine synthase (OASTL) genes have now been cloned from a range of plant species including spinach, wheat, *Arabidopsis*, *B. juncea*, rice, pepper, watermelon and potato (Römer et al., 1992; Rolland et al., 1993; Youssefian et al., 1993; Noji et al., 1994; Saito et al., 1994a; Barroso et al., 1995; Hesse and Hoefgen, 1998; Schäfer et al., 1998; Nakamura et al., 1999; Harada et al., 2001). Those that are located in the cytoplasm, chloroplasts and mitochondria are known as CSase A, B and C respectively (Saito et al., 1994b). SAT has also been cloned from *Arabidopsis* and watermelon (Bogdonova et al., 1995; Saito et al., 1995; Roberts and Wray, 1996).

Once synthesised, cysteine can directly form part of a protein or become a precursor for methionine and glutathione. Methionine is a member of the aspartate-derived amino acids, which also comprises lysine, threonine and isoleucine (Rose and Last, 1994; Galili, 1995). In addition to being a protein constituent, methionine is activated by ATP to form *S*-adenosylmethionine (SAM) which is an ubiquitous compound involved in many other biochemical reactions. Although the main role of SAM is for use in methylation, it is also involved in the biosynthesis of certain polyamines and the plant hormone, ethylene. Polyamines have been associated with growth stimulation (Slocum et al., 1984), and ethylene regulates a wide range of growth, developmental and defence-related processes including initiation of fruit ripening, seed germination, vascular gel formation in response to vascular pathogens and induction of compounds involved in SAR (VanderMolen et al., 1983; Yang and Hoffman, 1984; Fluhr and Mattoo, 1996; Dong, 1998). To produce methionine, cystathionine γ-synthase catalyses the synthesis of cystathionine from cysteine and *O*-phosphohomoserine in the plastids (Wallsgrove et al., 1983; Ravenel et al., 1995a; Ravanel et al., 1998a). Cystathionine is then cleaved into homocysteine, pyruvate and ammonia by cystathionine β-lyase. This enzyme was originally found both in the plastids and the cytosol (Wallsgrove et al., 1983; Droux et al., 1995; Ravanel et al., 1998a) but only the plastidic form is now actually thought to play a role in methionine synthesis (Ravenel et al., 1995b; Ravenel, 1997; Turner et al., 1998). Finally methionine synthase catalyses the methylation of homocysteine to form

methionine in the cytosol (Eichel et al., 1995; Ravanel et al., 1998a). In plants neither cystathionine γ-synthase nor cystathionine β-lyase activity is significantly affected by feedback inhibition by methionine pathway intermediates or end products. However there are strong indications that there is control at the level of gene expression by methionine, and the major site for such regulation is cystathionine γ-synthase (Ravanel et al., 1998a). If the intracellular pool of methionine increases, the level of extractable cystathionine γ-synthase activity is reduced (Giovanelli et al., 1985). Furthermore, under conditions of methionine starvation there is a substantial increase in extractable cystathionine γ-synthase activity (Datko and Mudd, 1982; Thompson et al., 1982). All of the genes involved in methionine biosynthesis have been cloned in plants. Cystathione γ-synthase has been cloned from *Arabidopsis* (Kim and Leustek, 1996), cystathionine β-lyase from *Arabidopsis* and potato (Ravenel et al., 1995b; Maimann et al., 2000) and methionine synthase from *C. roseus*, *Coleus blumei*, and *Arabidopsis* (Eichel et al., 1995; Petersen et al., 1995; Ravanel et al., 1998b).

Cysteine is a precursor of what is often described as the most abundant non-protein thiol compound in plants, the tripeptide glutathione (GSH, γ-glutamyl-cysteinylglycine) (Hell, 1997). Biosynthesis of glutathione occurs in two steps. Firstly γ-glutamylcysteine is synthesised from glutamate and cysteine by γ-glutamylcysteine synthetase. Following this, glycine is added to the C-terminus site of γ-glutamylcysteine by glutathione synthetase to yield glutathione (May et al., 1998; Noctor et al., 1998a). Both of these enzymes are found in both the cytosol and plastids but not in the mitochondria (Hell and Bergmann, 1988; Hell and Bergmann, 1990; Rüegsegger and Brunold, 1993). Cysteine pools are often tightly controlled depending on the availability of reduced sulphur products and OAS (as previously discussed). Adding cysteine to plants often enhances glutathione synthesis suggesting integration between cysteine pools and glutathione synthesis (Farago and Brunold, 1994; Schneider and Bergmann, 1995; Strohm et al., 1995; Noctor et al., 1996). Photorespiratory glycine is also essential for synthesis of glutathione (Buwalda et al., 1990; Noctor et al., 1997; Noctor et al., 1999). To investigate the regulation of glutathione synthesis further, poplar plants were transformed to overexpress either γ-glutamylcysteine synthetase or glutathione synthetase, and *Arabidopsis* plants with mutated γ-glutamylcysteine synthetase genes (*cad2-1* mutants) were also investigated (Strohm et al., 1995; Noctor et al.,

1996; Cobbett et al., 1998; Noctor et al., 1998b). These studies suggested that control of glutathione synthesis was a multistep procedure that included not only the availability of cysteine, but also the availability of γ-glutamylcysteine and the activity of glutathione synthetase. The availability of γ-glutamylcysteine may be controlled by feedback inhibition of γ-glutamylcysteine synthetase by glutathione, although this inhibition can be overcome (Brunold and Rennenberg, 1997; May et al., 1998). Evidence has also been presented that glutathione, like cysteine, homeostatically regulates sulphur nutrition through its effects on sulphate uptake and assimilation (Herschbach and Rennenberg, 1991; Herschbach and Rennenberg, 1994; Lappartient and Touraine, 1996; Smith et al., 1997; Takahashi et al., 2000). Both γ-glutamylcysteine synthetase and glutathione synthetase genes have been cloned in plants. γ-glutamylcysteine synthetase has been cloned from *Arabidopsis, B. juncea* and tomato (May and Leaver, 1994; Kovari et al., 1997; Schäfer et al., 1998) and glutathione synthetase has been cloned in *Arabidopsis, B. juncea, Medicago truncatula* and tomato (Rawlins et al., 1995; Ullmann et al., 1996; Schäfer et al., 1998; Skipsey et al., 1999; Frendo et al., 2001; I. Kovari and P. Goldsbrough, Purdue University, Indiana, USA, unpublished data).

Glutathione has many roles in plants. As well as being able to regulate sulphate uptake and xylem loading in the roots, it is also a major storage form of reduced sulphur and its mobility enables the long distance transport of reduced sulphur compounds to individual organs according to their requirements (De Kok and Stulen, 1993; Brunold and Rennenberg, 1997). Glutathione also has an important role in plant defence against both abiotic and biotic stresses which, due to the nature of this project, will be considered in more detail below (see also De Kok and Stulen, 1993; Lamoureux and Rusness, 1993; Marrs, 1996; Foyer et al., 1997; Kömives et al., 1998; May et al., 1998; Noctor et al., 1998a; Edwards et al., 2000; Tausz and Grill, 2000; Cobbett et al., 2001; Gullner and Kömives, 2001; Hall, 2002).

Some heavy metals are essential in plants as cofactors in redox reactions and ligand interactions. However if their concentrations become too high they may become toxic for plants by displacing metal cofactors from their cellular binding sites or undergoing aberrant reactions with thiol groups of proteins and coenzymes (Stadtman, 1990). Glutathione itself may participate directly in the chelation of

metal ions in plant cells at low heavy metal concentrations (Vogeli-Lange and Wagner, 1996). However, glutathione can also be polymerised to form phytochelatins containing 2 to 11 units (Rauser, 1995; Zenk, 1996; Cobbett, 2001; Hall, 2002). This is carried out in the cytosol by the enzyme phytochelatin synthase (Grill et al., 1989). Phytochelatins have a high affinity for metals such as cadmium and copper, to which they bind. These complexes are then transported to the vacuole where the metals are sequestered away from any sensitive plant components (Rauser, 1990; Cobbett, 2001; Hall, 2002). Phytochelatin synthase is expressed constitutively in plants but may be rapidly activated by heavy metals (Zenk, 1996; Cobbett, 2001). This rapid activation may provide plants with a level of resistance to heavy metals as plants which have a reduced capacity to produce phytochelatins are hypersensitive to the metals (Howden et al., 1995a; Howden et al., 1995b; Inouhe et al., 2000; Vernoux et al., 2000). Phytochelatin synthase has now been cloned from *Arabidopsis* (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999). Although many studies support a role for phytochelatins in heavy metal tolerance, particularly for cadmium, several studies do not (Cobbett, 2001; Hall, 2002). Therefore it has been suggested that phytochelatins may also play other roles in plant cells, including heavy metal homeostasis, sulphur metabolism or as antioxidants (Rauser, 1995; Dietz et al., 1999; Cobbett, 2000).

Glutathione is also involved in the detoxification of toxic organic compounds. Many hydrophobic and electrophilic toxins can be reacted with glutathione by a family of glutathione *S*-transferases (GSTs) that are produced constitutively in plants (Edwards et al., 2000). The *S*-glutathionylated metabolites are then tagged for vacuolar import where they are rapidly metabolised (Lamoureux and Rusness, 1993; Lu et al., 1997; Rea et al., 1998). Numerous plants have been found to express GSTs including wheat, tobacco, tomato, maize and *Arabidopsis* (Marrs, 1996; Edwards et al., 2000).

Oxidative stress is an inescapable feature of life for plants, as ROS are involved in nearly all effects of environmental stresses. Stress impacts, such as extremes of temperature (Doke et al., 1994; Prasad et al., 1994; Kocsy et al., 2001), drought (Dhindsa, 1991; Smirnoff et al., 1993; Sgherri and Navari-Izzo, 1995), high light intensities (Foyer et al., 1994; Karpinski et al., 1997), heavy metals (Cakmak and Marschner, 1988; Foyer et al., 1994; Weckx and Clijsters, 1996; Dietz et al.,

1999), herbicides (Foyer et al., 1994), mechanical and physical stresses (Legendre et al., 1993), air pollutants (Foyer et al., 1994), and challenges by pathogens (the oxidative burst) (Bolwell and Wojtaszek, 1997; Lamb and Dixon, 1997; Wotjtaszek, 1997; Bolwell, 1999) may all induce the production of ROS such as hydrogen peroxide, superoxide and hydroxide radicals in plants. If these ROS are not detoxified, they cause severe damage to cellular components including nucleic acids, proteins and lipids (Imlay and Linn, 1988; Halliwell and Gutteridge, 1989). Plants contain many compounds and enzymes such as ascorbate, carotenoids, superoxide dismutases, peroxidases and catalases that can quench or inhibit formation of ROS and free radicals (Bolwell, 1996; Noctor and Foyer, 1998). Glutathione is also thought to play a role in this protection (De Kok and Stulen, 1993; Foyer et al., 1997; Kömives et al., 1998; May et al., 1998; Noctor et al., 1998a; Tausz and Grill, 2000; Gullner and Kömives, 2001).

Levels of glutathione and ascorbate are generally increased in plants in response to oxidative stress and correlate well with enhanced tolerance (Noctor et al., 1998a; Tausz and Grill, 2000). Often glutathione levels are found to compensate for other antioxidants, for example in catalase-deficient mutants or where catalase activity is decreased by antisense technology (Smith et al., 1984; Smith, 1985; Willekens et al., 1997). Furthermore when glutathione is depleted, increases in sensitivity to oxidative stress have been found (Kushnir et al., 1995). In the case of the oxidative burst, the accumulation of ROS is likely to contribute to the prevention and spread of the pathogen, either directly through their toxicity or role in cell death, or indirectly through signalling further defence mechanisms (chapter 1). However plant cells need to avoid excessive oxidative damage of the non-invaded plant tissues around the pathogen entry site in order to provide an effective defence response, and this is thought to be aided by GSH-dependent antioxidative processes (Kömives et al., 1998; Gullner and Kömives, 2001).

Glutathione can be oxidised to form GSSG (oxidised glutathione), which is returned to GSH by the NADPH-dependent enzyme, glutathione reductase, so that under non-stress conditions more than 90% is in the reduced form (Smith et al., 1989; Noctor et al., 1998a). GSH is a strong reductant and can directly detoxify ROS (Allen, 1977; Wefers and Sies, 1983; Larson, 1988; Winterbourn and Metodiewa, 1999), although it is much less efficient at detoxifying hydrogen peroxide than other mechanisms (Noctor et al., 1998a; Tausz and Grill, 2000). For

detoxification of hydrogen peroxide, GSH is more important in the regeneration of oxidised ascorbate in the ascorbate/GSH cycle (Noctor and Foyer, 1998; Noctor et al., 1998a; Foyer and Rennenberg, 2000; Tausz and Grill, 2000). Ascorbate is the most important reducing substrate for hydrogen peroxide detoxification in plants particularly in the chloroplast where there is a lack of catalase (De Kok and Stulen, 1993; Mehlhorn et al., 1996). For either of the mechanisms of ROS detoxification mentioned here, sustaining the high GSH/GSSG ratio is crucial. Glutathione reductase is therefore a key enzyme in defence against oxidative stress. It is found mainly in the chloroplasts although there are also cytosolic and mitochondrial forms (Edwards et al., 1990) and it has been cloned in plants such as pea, tobacco, rice and *Arabidopsis* (Criessen et al., 1992; Creissen and Mullineaux, 1995; Stevens et al., 1997; Kaminaka et al., 1998; Stevens et al., 2000). Overproduction of glutathione reductase particularly in the chloroplast has been shown to provide additional protection against oxidative stress (Aono et al., 1993; Aono et al., 1995a; Broadbent et al., 1995; Foyer et al., 1995). Increased sensitivity was reported in tobacco antisensed for glutathione reductase (Aono et al., 1995b).

As well as playing a role in the regulation of the oxidative burst, glutathione may also function in other aspects of plant defence against pathogens. GSH may assist in the rapid toughening of plant cell walls that occurs during the initial stages of plant attack to increase the effectiveness of this structural barrier (Bolwell, 1993; Gullner and Kömives, 2001). The addition of exogenous glutathione can elicit the transcription of defence genes encoding cell wall HRGPs (Wingate et al., 1988). Furthermore GSH is thought to interact with the ROS that mediate the covalent cross-linking of cell wall components and may regulate this process (Foyer et al., 1997; Bowell, 1999; Grant and Loake, 2000). GSH has also been found to induce the activities of enzymes participating in phytoalexin biosynthesis in bean and soybean cells. It was shown to activate the transcription of chalcone synthase and phenylalanine ammonia lyase (PAL) genes (Dron et al., 1988; Wingate et al., 1988). Chalcone synthase is involved in the formation of isoflavonoid phytoalexins (Hahlbrock and Scheel, 1989) and PAL is the key enzyme of phenylpropanoid biosynthetic pathway, which is involved in lignin and phenolic phytoalexin biosynthesis (Hahlbrock and Grisebach, 1979). The activation of genes involved in both cell wall strengthening and phytoalexin biosynthesis by GSH suggests that it may elicit signal transduction pathways leading to defence responses following

infection. However the artificial elevation of GSH levels by L-2-oxothiazolidine-4-carboxylic acid (OTC, a synthetic cysteine precursor) did not elicit phytoalexin biosynthesis. Furthermore the changes in GSH levels occurred too slowly to be involved in the initiation of the elicitation response. This has led to the suggestion that GSH plays a role solely in protection against excessive oxidative damage in plant defence (Edwards et al., 1991). However, phytoalexin accumulation can be induced by both increasing and decreasing the GSH levels in plants (Guo et al., 1993; Gullner and Kömives, 2001). It is possible that changes in the GSH/GSSG ratios or redistribution of thiols in various internal pools act as signals for elicitation. In spite of numerous studies showing that many plants infected by pathogens show substantial alterations in endogenous GSH levels, the exact role of GSH in elicitation of defence responses remains elusive (Kömives et al., 1998; Gullner and Kömives, 2001).

Infection of plants by pathogens markedly influences the activities of glutathione-related enzymes. GST activity can be induced in plants in response to fungal or bacterial infection or elicitation (Mauch and Dudler, 1993; Levine et al., 1994; El-Zahaby et al., 1995; Adám et al., 1997). Their role in plant defence may be linked to suppression of necrotic disease symptoms by the detoxification of toxic lipid hydroxyperoxides that derive from peroxidation of the cell membrane, thereby controlling the extent of hypersensitive cell death at the infection site (Gullner and Kömives, 2001). Glutathione reductase activity has also been associated with plant defence. Inoculation of tomato leaves with the fungal pathogen *Botrytis cinerea* led to a significant increase in glutathione reductase activity and this induction was coincident with the appearance of disease symptoms (Kuzniak and Sklodowska, 1999). Elevated glutathione reductase activity is thought to play a role in conferring more efficient antioxidant protection to infected tissue by maintaining the high GSH/GSSG ratio (Gullner and Kömives, 2001).

Based on information as to how sulphur is assimilated in plants (summarised in Fig. 4.1), predictions may be made on the biosynthetic origin of elemental sulphur. A prerequisite would be to investigate how normal sulphur metabolism is affected by pathogen infection using resistant tomato plants challenged with *Verticillium dahliae* as the model. This may provide clues as to the origin of the sulphur. Investigations have been carried out firstly by Northern analysis using some of those genes involved in sulphur assimilation that have been cloned in tomato, or

its close relative potato, as probes. Secondly in collaboration with J. Howarth (IACR Rothamsted, Harpenden, UK) screening techniques (production of a suppression subtractive library and cDNA-Amplified Fragment Length Polymorphism (AFLP) analysis) have been used to isolate clones that are up-regulated during the resistance response. It was thought that cDNA-AFLP would yield fewer false positives and that production of a suppression subtractive library would be better at amplifying any low abundant transcripts that are up-regulated (M. Hawkesford pers. comm., IACR Rothamsted, Harpenden, UK) and therefore both techniques were used in parallel.

4.2 Materials and Methods

4.2.1 Plant material

Resistant tomato plants (cultivar GCR 218) were grown and inoculated either with *Verticillium dahliae* or sterile water and the stems harvested up to node 8 for xylem as described previously (3.2.1, 3.2.2 & 3.2.6). Three control plants and three pathogen-inoculated plants were harvested immediately following inoculation (0 dpi) and then at the same time points used previously for elemental sulphur analysis (7, 14 and 21 dpi). At 14 dpi (elemental sulphur detected in resistant challenged plants) a further three *V. dahliae*-inoculated and control plants were harvested, but xylem was removed from the plant in three sections: from the base of the stem to node 4, nodes 5 to 9 and nodes 10 to 14. At 21 dpi, a sample of leaves was also harvested from a control plant which was to be used as a positive control for those genes that had been previously been found to be expressed in leaf tissue (M. Hawkesford pers. comm., IACR Rothamsted, Harpenden, UK). Xylem and leaf samples harvested from individual plants or individual sections of the same plant were wrapped separately in aluminium foil. All samples were immediately frozen in liquid nitrogen.

The first batch of xylem samples harvested at 0, 7, 14 and 21 dpi and removed up to node 8 together with the leaf sample, were stored at the University of Bath (Bath, UK) at -70°C until use. These samples were used to determine changes in normal plant sulphur metabolism in response to a challenge by *V. dahliae* by Northern analysis. The second and third batches of xylem harvested from the base of tomato plants up to node 8 at 0, 7, 14 and 21 dpi, and the xylem harvested from plants split into three sectors at 14 dpi were sent to J. Howarth (IACR Rothamsted, Harpenden, UK) on dry ice. This material was used in differential display techniques to determine genes up-regulated in response to the pathogen as well as for subsequent Northern analyses.

4.2.2 Probes for Northern analysis

The probes used for Northern analysis are described in detail in Table 4.1 below. All but two are genes of normal plant sulphur metabolism, cloned from either tomato or potato and sent from various sources. G7-3 was isolated from tomato by suppression subtractive hybridisation. 18S rRNA was cloned from wheat but is similar enough to that of tomato to confirm equal loading on Northern blots.

Name of probe	Function or putative function of probe	Vector	Size (bp)	Source	Other Information
LeST2	Tomato sulphate transporter	pGEM-T Easy (Promega, Southampton, UK)	1020	J. Howarth (IACR, Rothamsted, Harpenden, UK)	Fragment of Accession no. AF347614 (bases 418-1438) J. Howarth and M. Hawkesford (IACR Rothamsted, Harpenden, UK, unpublished data)
APSR	Potato APS reductase	pGEM-T Easy	1000	L. Hopkins (IACR, Rothamsted, Harpenden, UK)	L. Hopkins and M. Hawkesford (IACR Rothamsted, Harpenden, UK, unpublished data)
G7-3	Tomato gene of unknown function	pGEM-T Easy	292	J. Howarth (IACR, Rothamsted, Harpenden, UK)	Product of a cDNA subtractive library with a short domain showing homology to rhodanese genes (sulphurtransferases)
StCS-A	Potato cytosolic cysteine synthase	pBluescript (Stratagene, Amsterdam, The Netherlands)	1308	H. Hesse (Max Plank Institute, Golm, Germany)	Complete cDNA Accession no. AF044172 Hesse and Hoefgen (1998)
StCS-B	Potato plastidic cysteine synthase	pBluescript	1404	H. Hesse (Max Plank Institute, Golm, Germany)	Complete cDNA Accession no. AF044173 Hesse and Hoefgen (1998)
Tom-GSH2/3	Tomato gluthathione synthetase	pBluescript	674	P. Goldsbrough (Purdue University, Indiana, USA)	5' untranslated region plus first 239 bases of cDNA Accession no. AF017984 Note, the whole sequence has been cloned but was found to give non-specific binding and so the 674 bp fragment was used P. Goldsbrough pers. comm. (Purdue University, Indiana, USA).
Wheat 18S rRNA	Wheat 18S rRNA	pGEM-T Easy	1000	J. Howarth (IACR, Rothamsted, Harpenden, UK)	P. Buchner and M. Hawkesford (IACR Rothamsted, Harpenden, UK, unpublished data)

Table 4.1 Source and details of DNA used as probes for Northern analysis.

4.2.3 PCR amplification of DNA probes

All probes were sent in vectors in TE buffer (appendix A2.1). Prior to PCR amplification, probes were diluted 1 in 10 with sterile milli-Q water. A PCR reaction master mix was produced for 5 reactions consisting of 62.5 μL REDTaq™ ReadyMix™ (Sigma, Poole, UK), 10 μL forward primer (50 mM stock), 10 μL reverse primer (50 mM stock), 37.5 μL water (supplied with REDTaq). The primers used for amplification are described in detail in Table 4.2 below.

Primer name	Vector to be used with	Forward/ Reverse	Sequence (5' – 3')
M13	pGEM-T Easy	Forward	GTTTTCCCAGTCACGAC
M13	pGEM-T Easy	Reverse	CAGGAAACAGCTATGAC
T7	pBluescript	Forward	TAATACGACTCACTATAGGG
T3	pBluescript	Reverse	ATTAACCCTCACTAAAGGGA

Table 4.2 Details of primers used for amplification of DNA cloned into either pGEM-T Easy or pBluescript vectors.

For each reaction 24 μL of master mix was pipetted into each PCR tube and 1 μL DNA added. Tubes were placed in a PTC-100™ PCR machine (MJ Research Inc., Nevada, USA) and PCR was carried out with the following conditions;

Step 1: 95°C for 2 min
Step 2: 95°C for 30s
Step 3: 55°C for 30s
Step 4: 72°C for 1 min
Step 5: Go back to step 2
Step 6: Repeat step 5 35 times
Step 7: 72°C for 5 min

PCR products were then visualised by agarose gel electrophoresis.

4.2.4 Agarose gel electrophoresis

1% agarose gels were prepared in 1X TBE buffer (appendix A2.1; A2.2) and the agarose dissolved by heating. When the gel had cooled to ≤60°C, ethidium bromide was added to a final concentration of 50 μg/mL. The mixture was then poured into a gel tray to produce gels of approximately 1 cm thickness. A comb was used to form the wells. Once the gel had set it was placed in a gel tank containing 1X TBE buffer. If required, samples were mixed with 1/5 volume loading

buffer (appendix A2.1; A2.2) and pipetted into the wells. PCR products produced with REDTaq™ did not require the addition of loading buffer as it was already present within the readymix. A 1 kb DNA ladder (New England BioLabs Inc., Hitchin, UK) was used as a molecular wt marker. Electrophoresis was carried out at 100V for 30 min to 2h. Bands of DNA or RNA were visualised under UV light with a UV transilluminator (UVP, Cambridge, UK) and documented with a digital graphic printer (Sony, Brooklands, UK).

4.2.5 DNA purification from agarose gels

PCR products visualised as bands on agarose gels were removed with a razor blade, placed in a 1.5 mL microcentrifuge tube and weighed. Bands were then purified by a QIAquick gel extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Purified DNA was suspended in 50 μL milli-Q water, quantified and stored at -20°C.

4.2.6 Quantification of DNA

For quantification of DNA, 0.5 μg of a 1 kb DNA ladder (New England BioLabs Inc.) was used as a marker and run on a 1% agarose gel alongside a known volume of the DNA sample to be quantified. For each 0.5 μg loading of ladder, the length of the DNA in bp and the DNA mass for each band had been pre-calculated by the manufacturers. Sample DNA concentration was then estimated by comparison of band intensity with a marker band of a similar length.

4.2.7 RNA extraction

Extraction of total RNA from samples of tomato xylem was attempted firstly using RNeasy Plant Mini Kit (Qiagen) according to the instructions of the manufacturer but this did not yield enough RNA for multiple Northern blots (only 4 to 15 μg RNA per spin column was obtained). Therefore RNA was extracted from both xylem and leaf samples according to a method adapted from that of Leech et al. (1998). All glassware used for RNA extraction was baked at 180°C overnight and all solutions were made up with diethyl pyrocarbonate (DEPC)-treated (RNAse free) milli-Q water (appendix A2.2) and autoclaved to prevent RNA degradation by RNAses. Each sample was comminuted to a fine powder in liquid nitrogen in a clean pestle and mortar previously cooled to -70°C. Liquid nitrogen was continuously added to the sample to prevent thawing and subsequent degradation

of RNA by RNAses in the plant tissues. 3 to 4g were transferred into a pre-cooled 50 mL centrifuge tube containing 10 mL TLES buffer (appendix A2.2) and 10 mL aqueous saturated phenol (Fluka, Poole, UK) with a pre-cooled spatula (pre-cooling was achieved by dipping the item in liquid nitrogen). The sample was immediately vortexed for 1 min, 10 mL of chloroform:isoamyl alcohol (24:1 v/v) added, the sample vortexed for a further min and then left to stand whilst the other samples were comminuted. All samples were then centrifuged for 5 min at 1,000*g* and the upper phase carefully removed to a fresh 50 mL centrifuge tube where an equal volume of phenol:chloroform:isoamylalcohol (25:24:1 v/v) was added. Tubes were again vortexed for 1 min, centrifuged for 5 min at 1,000*g* and the upper phase carefully transferred to a 30 mL Corex® tube. An equal volume of 4M LiCl (appendix A2.2) was added, the top of the Corex tube sealed with parafilm and the contents of the tubes mixed by inversion. Tubes were then left overnight at 4°C to precipitate the RNA.

Following precipitation, samples were centrifuged at 12,000*g* for 30 min at 4°C. The supernatant was discarded and the RNA pellet resuspended in 1 mL of RNAse-free milli-Q water by gently pipetting the solution up and down several times with a pasteur pipette. The RNA was then ethanol-precipitated to remove any LiCl (4.2.8) and the samples centrifuged at 12,000*g* for 20 min at 4°C. The pellet was then washed to removed any salt by resuspending it in 5 mL 70% ethanol (appendix A2.2) and centrifuging again at 12,000*g* for 20 min. As much ethanol as possible was removed by pasteur pipette and the remainder evaporated by baking at 65°C for 2 to 3 min. The RNA pellet was then dissolved in 500 μL RNAse free milli-Q water and stored at -70°C. An aliquot of each RNA sample was run on a 1% agarose 1X TBE gel (4.2.4) and also assessed spectrophotometrically at A_{260} and A_{280} to test the quality and quantity of RNA. The quality of the RNA was good and the quantity ranged from 60 to 300 μg of total RNA per sample.

4.2.8 Ethanol precipitation of RNA

In order to ethanol precipitate RNA, 0.1 volumes of 3M sodium acetate (appendix A2.2) and 2.5 volumes of absolute alcohol (Fisher, Loughborough, UK) were added to the sample and the RNA placed at -20°C overnight.

4.2.9 Standardising the concentration of aqueous solutions of RNA

As the concentrations of aqueous solutions of RNA varied quite considerably between samples, the concentrations were standardised prior to formaldehyde gel electrophoresis. 20 μg aliquots of RNA from each sample were ethanol-precipitated as described previously (4.2.8). Samples were then centrifuged at 15,800*g* (5415C Eppendorf, New York, USA) for 15 min, the supernatant discarded and the pellet washed in 500 μL of 70% ethanol. The RNA aliquots were then centrifuged again at 15,800*g* for 15 min and the ethanol removed. The pellets were dried at 65°C for 5 to 10 min and then resuspended in 20 μL of RNAse free milli-Q water.

4.2.10 Formaldehyde gel electrophoresis of RNA

Prior to electrophoresis the gel tank, tray and comb were soaked overnight in milli-Q water containing 0.01% DEPC. The gel apparatus was then rinsed in RNAse free milli-Q water. The formaldehyde gel was prepared by adding 3g of agarose to 150 mL of RNAse free milli-Q water and heating to dissolve the agarose. Once the gel had cooled to approximately 65°C, 20 mL of 10X MEN buffer (appendix A2.2) and 32 mL of formaldehyde (Prolabo, Merck Eurolab, France) were added. The gel was then mixed thoroughly and poured into a gel tray that had previously been made level with a spirit level to produce a gel of uniform thickness. The gel was then allowed to set for at least 1h.

A master mix of loading buffer was prepared by mixing 240 μL formamide (Sigma), 72 μL formaldehyde, 60 μL 10X MEN buffer and 1 μL ethidium bromide (the values above were calculated for 12 RNA samples instead of 9 to allow for pipetting error). 31 μL of this master mix was added to each 20 μg RNA sample and the contents mixed by vortexing. The RNA was then denatured at 65°C for 15 min, placed immediately on ice for 5 min and briefly centrifuged.

The formaldehyde gel was placed in the gel tank with 1X MEN as the tank buffer (appendix A2.2). Tank buffer was added until it just reached the top of the gel. The gel was then loaded with the RNA samples. 5 μL of loading buffer (appendix A2.2) was loaded into an empty outside lane in order to determine how far the gel had run. The gel was then run at 70V for approximately 4h and visualised under UV

light with the UV transilluminator to confirm the quality and equal loading of the RNA.

4.2.11 Northern Blotting

Again all glassware for producing a Northern blot was pre-treated to remove RNAses either by baking at 180°C overnight, or soaking in a solution of 0.1M NaOH 1 mM EDTA (appendix A2.2) and then rinsing with RNAse free milli-Q water. The gel was soaked in milli-Q water for 15 min at room temperature to remove excess formaldehyde and then washed for 15 min in 10X SSC (appendix A2.2) twice.

The Northern blotting apparatus was set up as follows: a large tray was filled with 10X SSC and a plastic gel tray was inverted and placed into the liquid. Two sheets of chromatography paper grade FN100 (Sartorius, Goettingen, Germany) were cut and draped over the inverted gel tray into the 10X SSC to form a wick. Once the chromatography paper had become soaked in the 10X SSC any air bubbles were removed by rolling over it with a freshly opened sterile 10 mL graduated pipette (Greiner, Stonehouse, UK). The gel was placed onto the chromatography paper and a piece of Hybond™-XL nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) cut to approximately the same size as the gel placed on top, again taking care to avoid air bubbles. Once again a sterile pipette was rolled over the nylon membrane to ensure full contact with the gel. Saran wrap (Dow, Edegem, Belgium) was placed over the whole tray and the region covering the gel removed using a razor blade. This was to prevent evaporation of the SSC and "short circuiting" of the SSC around the gel instead of through it. Three more pieces of chromatography paper were placed on top of the nylon membrane followed by 8 to 10 cm of blue paper towels (Lotus, Sheffield, UK) and finally a glass plate. This apparatus was left overnight to allow the RNA to migrate and attach to the membrane. The nylon membrane was then removed, placed face down onto a piece of Saran wrap and exposed to UV on a UV transilluminator for 2 min to fix the RNA to the membrane. The lanes of RNA were marked on the membrane with a pencil in order to determine in which sample changes in gene expression had occurred in later hybridisations. The gel was also examined under UV to confirm that no RNA remained. Membranes were either used immediately for hybridisation or were placed in Saran wrap with 1 mL 2X SSC 0.1% sodium dodecyl sulphate (SDS) (appendix A2.2) and stored at 4°C.

4.2.12 Radiolabelling of probes and Northern hybridisation

Each membrane was rolled up (RNA facing inwards) placed in a glass hybridisation tube (HB-OV-BL, Hybaid, Ashford, UK) and approximately 15 mL of hybridisation buffer (appendix A2.2) added. Membranes were then pre-hybridised in a hybridisation oven (Hybaid) for at least 1h at 61°C for those probes originating from potato and wheat and at 65°C for probes originating from tomato.

Previously amplified, gel purified and quantified DNA was used to produce radiolabelled probes with an Oligolabelling Kit (Amersham Pharmacia Biotech). 25 to 50 ng of DNA suspended in milli-Q water was made up to 34 μL with TE buffer. DNA was then denatured for 3 to 4 min at 100°C and immediately placed on ice for 2 min. 10 μL of reagent mix (provided in the oligolabelling kit), 5 μL of [α-^{32}P]dCTP (ICN, Basingstoke, UK) and 1 μL of Klenow fragment (provided in the oligolabelling kit) was then added to the DNA and the tube incubated at 37°C for 60 min. During this time a Sephadex G-50 column was prepared. A small piece of cotton wool was pushed to the end of a 2 mL syringe barrel (Terumo, Leuven, Belgium) in order to plug it. Sephadex™ G-50 Fine (Amersham Pharmacia Biotech) suspended in STE buffer (appendix A2.2) was applied to the top of the syringe barrel and compacted with the plunger until a volume of 2 mL of Sephadex was present. The column was then washed by passing 3 mL of STE buffer (appendix A2.2) through it.

After the DNA and radiolabelled nucleotides had incubated for 60 min, 150 μL of STE buffer was added. The solution was then passed through a Sephadex column to remove any unincorporated nucleotides and the eluted radiolabelled DNA collected and denatured by heating at 100°C for 3 to 4 min. The radiolabelled probe was then dispensed into the hybridisation tube containing the pre-hybridised membrane, and hybridisation allowed to proceed overnight.

Following hybridisation membranes were washed in two 10 min washes of 2X SSC 0.1% SDS (appendix A2.2) at 55°C for potato and wheat probes and 65°C for tomato probes. Membranes were removed from hybridisation tubes and the intensity of radioactivity checked with a Geiger counter. If the counts per s were above 10 the membrane was given an extra 10 min wash in 1X SSC 0.1% SDS (appendix A2.2). Membranes were then wrapped in Saran wrap and secured into

an autoradiography cassette with an intensifying screen. Autoradiography film (Kodak, New York, USA) was placed on top of the membrane and the cassette stored at -70°C. Autoradiographic films were processed with a Hyperprocessor (Amersham Pharmacia Biotech). All probes listed in Table 4.1 were tested by Northern analysis. The 18S rRNA probe from wheat was used to confirm equal loading of RNA on the membrane.

4.2.13 Stripping of Northern blots

Northern blots could be reused 3 to 4 times. Probes were removed from the blots by soaking them in twice in boiling 0.1% SDS (appendix A2.2) for 30 min. The 18S rRNA probe was difficult to remove and therefore was used last.

4.2.14 Summary of techniques to clone differentially expressed genes (performed by J. Howarth, IACR Rothamsted, Harpenden, UK)

Xylem material harvested from the bottom sector (base of stem to node 4) of *V. dahliae*-inoculated and control tomato plants at 14 dpi was sent to J. Howarth for cDNA-AFLP analysis. J. Howarth also produced a subtractive cDNA library from xylem harvested from control and *V. dahliae*-challenged tomato plants grown, inoculated and harvested at IACR Rothamsted. Both techniques were used to isolate genes up-regulated in tomato in response to the pathogen.

For cDNA-AFLP analysis, cDNA was synthesised from mRNA using the SMART cDNA synthesis kit (Clontech Laboratories Inc, Oxford, UK). cDNA was then digested with *Mse* I and *Eco* RI, ligated to adapter primers of known sequence (listed below) and AFLP performed according to Bachem et al. (1996) and Vos et al. (1995) using primers homologous to the adaptor sequences but with a three-nucleotide extension.

Mse I Adapter primer 1:	5'-GACGATGAGTCCTGAG-3'
Mse I Adapter primer 2:	5'-TACTCAGGACTCAT-3'
Eco RI Adapter primer 1:	5'-CTCGTAGACTGCGTACC-3'
Eco RI Adapter primer 2:	5'-AATTGGTACGCAGTCTAC-3'
Mse I Preamplification primer:	5'-GACGATGAGTCCTGAGTAA-3'
Eco RI Preamplification primer:	5'-CTCGTAGACTGCGTACCA-3'

Gene expression levels were visually compared by running AFLPs from pathogen-infected and control samples side by side on polyacrylamide sequencing gels and exposing them to autoradiography film (Bachem et al., 1996). Gene fragments were resolved to one bp size difference on gels. 52 primer pairs were used in the comparison of infected and control material and the expression of 200 to 300 fragments were compared for each primer pair. Therefore approximately 10400 to 15600 fragments in total were compared in this study. Bands that showed increased intensity in the infected tissue compared to the control were cut out of the gel for subcloning and sequence analysis.

Suppression subtractive hybridisation was carried out using a CLONTECH PCR-Select™ cDNA subtraction kit (Clontech Laboratories Inc.), according to the manufacturer's instructions. A PCR-Select™ differential screening kit was then used to detect false positive clones (clones amplified during cDNA subtraction which were not differentially expressed between the sample mRNA pools). Clones remaining were subject to sequence analysis.

Reverse Northern blot analysis was carried out on all clones isolated by suppression subtractive hybridisation and cDNA-AFLP analysis to reduce further false positives. Radiolabelled antisense strand cDNA probes were made from pathogen-challenged and control RNA harvested from the base of the plant at 14 dpi. These probes were used to hybridise against replicate blots each containing approximately 1 μg of each potentially up-regulated clone.

Clones that were shown to be up-regulated by the reverse Northern screening stage were then subjected to a further screen by Northern analysis. Xylem harvested from the base of the plant up to node 8 at 0, 7, 14 and 21 dpi and xylem harvested from the three different sectors (base, middle and top) of the plant at 14 dpi from both *V. dahliae*-challenged and control tomato plants were used for Northern analysis. This confirmed up-regulation of selected clones and determined the time course of expression during the challenge. One clone from the subtractive cDNA library, G7-3, was sent to the University of Bath for Northern analysis. The remaining clones and G7-3 were also analysed by J. Howarth using infected and control xylem tissue sent from the University of Bath.

4.3 Results

4.3.1 Changes in the expression of genes involved in normal plant sulphur metabolism in resistant tomato plants challenged with *V. dahliae*

Gene expression in xylem tissue of resistant (cultivar GCR 218) tomato plants inoculated with either sterile water or *V. dahliae* was analysed by Northern analysis using the probes listed in Table 4.1. The autoradiographs produced from exposure to probed blots are shown in Fig. 4.2. RNA from control plant leaf tissue was also included on the blots and all probes were found to be expressed constitutively in leaf tissue.

The expression of the tomato sulphate transporter was shown to increase over the time course in both water-inoculated and pathogen-inoculated xylem. However expression appeared to increase more rapidly in the inoculated xylem as seen by a more intense band on autoradiography film at 7 dpi compared to control tissue. A similar pattern of gene expression was also evident with APS reductase and glutathione synthetase both of which showed up-regulation at 7 dpi in *V. dahliae*-inoculated xylem compared to control xylem.

Plastidic and cytosolic cysteine synthase probes both gave a similar pattern of expression. They showed a high level of expression at 0 dpi in both control and *V. dahliae*-inoculated xylem. In the control xylem expression decreased at 7 and 14 dpi recovering by 21 dpi but in *V. dahliae*-inoculated tissue it remained high, particularly at 7 dpi, but also at 14 dpi in the plastidic form.

4.3.2 Genes up-regulated in resistant tomato plants in response to *V. dahliae* as determined by suppression subtractive hybridisation or cDNA-AFLP analysis

cDNA-AFLP analysis followed by reverse Northern analysis yielded 14 clones which were potentially up-regulated in resistant tomato plants in response to *V. dahliae*. These were all analysed by Northern analysis by J. Howarth. All but one proved to be false positives. However one clone, designated 6-5, was shown to be up-regulated in response to *V. dahliae*. No expression of this clone could be detected in resistant plants prior to infection. However a strong and specific induction of expression was observed at 7, 14 and 21 dpi and at the base, middle and top of the plant at 14 dpi in response to pathogen infection (Fig. 4.3). The clone is a 120 bp fragment, and has no homology to any previously cloned genes

in the GenBank or The Institute of Genomic Research (TIGR) tomato expressed sequence tag (EST) databases.

Suppression subtractive hybridisation followed by reverse Northern analysis only yielded one potential clone that was up-regulated in resistant tomato plants in response to *V. dahliae*. This was named G7-3 and contained a short domain showing homology to rhodanese genes (sulphurtransferases). Due to its possible link with sulphur metabolism, this gene was analysed by Northern analysis both at the University of Bath and by J. Howarth at IACR Rothamsted. However G7-3 was found to be a false positive, as negligible differences in expression between the control and pathogen-inoculated xylem were detected over the time course (Fig. 4.2).

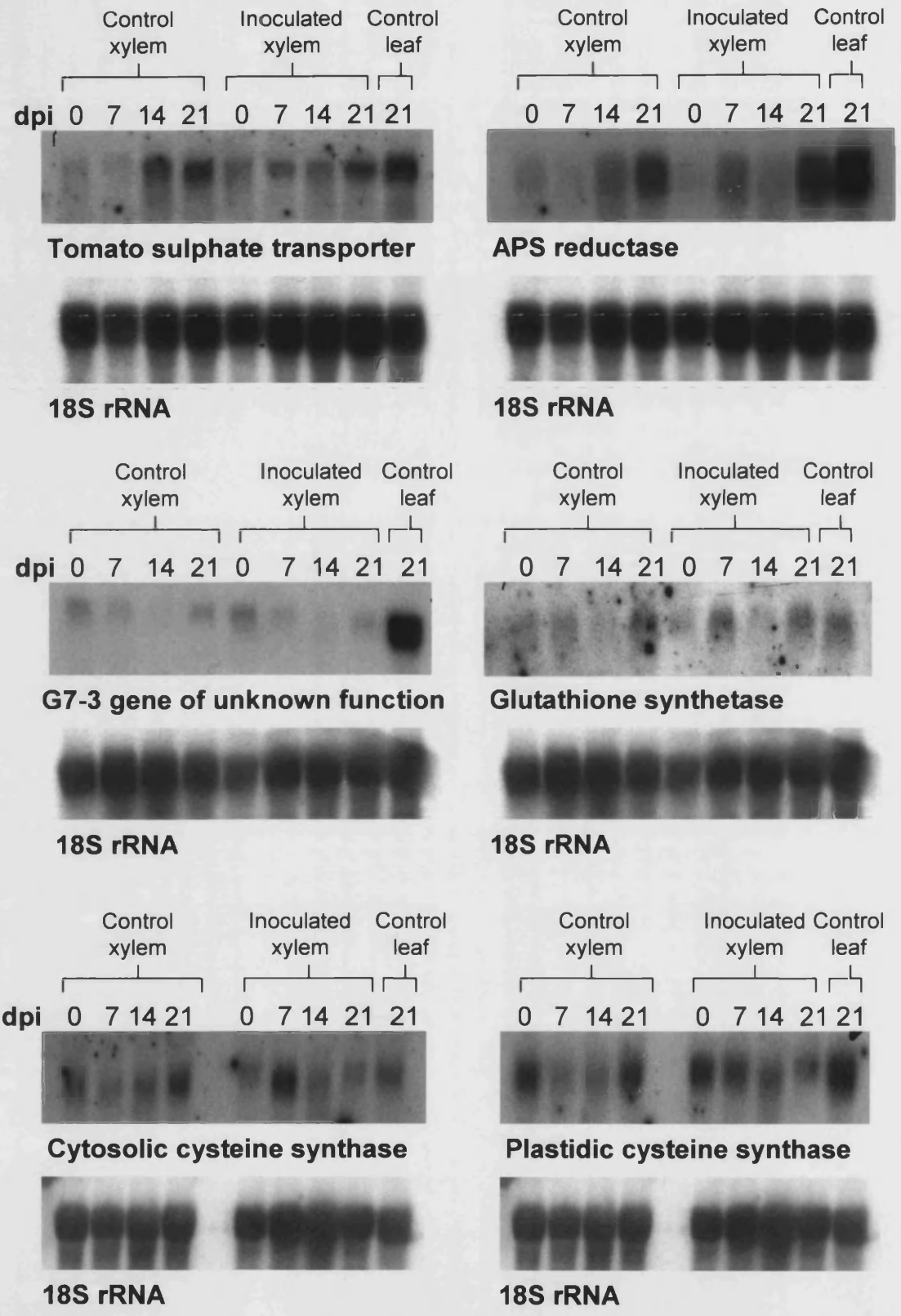

Figure 4.2 Gene expression in resistant tomato plants inoculated with *V. dahliae* as shown by Northern analysis. RNA was extracted from xylem harvested at 0, 7 ,14 and 21 dpi from the base to node 8 of tomato plants inoculated with either sterile water or *V. dahliae* and used to produce 20 μg Northern blots. Blots were probed with various genes believed to be involved in sulphur metabolism and with 18S rRNA to confirm equal loading. RNA from water inoculated tomato leaves was also included on the blot as a positive control. The images are autoradiographs exposed to blots hybridised with radiolabelled probes.

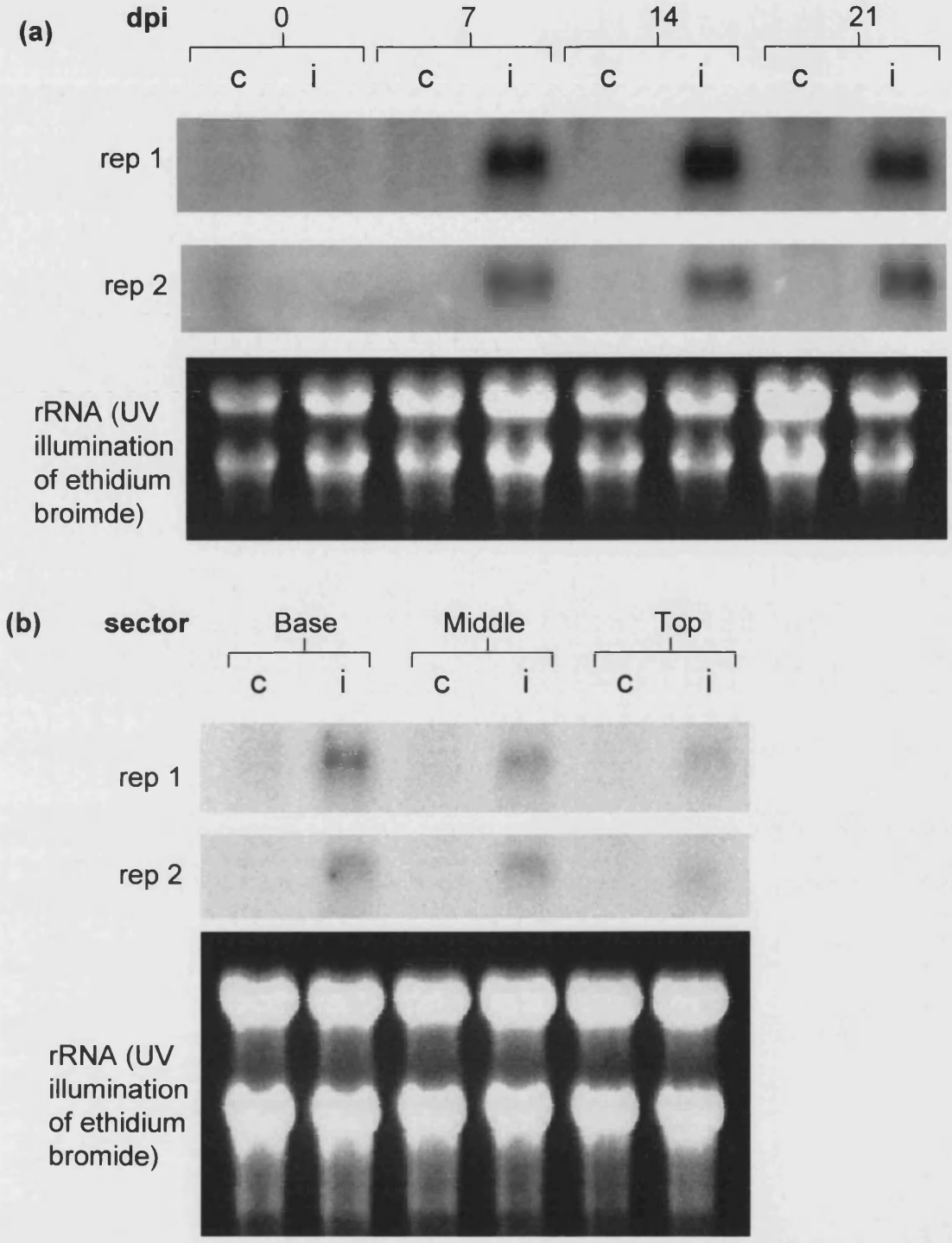

Figure 4.3 Expression of clone 6-5 in resistant tomato plants challenged with *V. dahliae* as shown by Northern analysis (performed by J Howarth, IACR Rothamsted, Harpenden, UK). Clone 6-5 was initially shown to be up-regulated in response to *V. dahliae* by cDNA AFLP analysis and by reverse Northern analysis. Up-regulation was confirmed by Northern analysis (a & b). RNA was extracted from xylem harvested from the base of the plant up to node 8 at 0, 7, 14 and 21 dpi (a) and from the three sectors (base to node 4 (base), nodes 5 to 9 (middle) and nodes 10 to 14 (top)) at 14 dpi (b) from both *V. dahliae*-challenged (i) and control (c) tomato plants. UV illumination of rRNA stained with ethidium bromide was used to confirm equal loading. No expression of clone 6-5 could be detected in resistant plants prior to infection. However strong induction could be detected at all other time points and in all three sectors of the plant in response to pathogen infection. This was confirmed with two replicate batches of xylem material.

4.4 Discussion

The origin and biosynthetic pathway of elemental sulphur production remains unresolved. However analysis of gene expression has provided an insight into the responses that occur on pathogen challenge.

An investigation carried out by S. Hall (IACR Rothamsted, Harpenden, UK) on the effect of *V. dahliae* infection on sulphate, glutathione, and cysteine levels in tomato tissues by HPLC was performed on the same xylem material used for the detection and quantification of elemental sulphur in tomato plants described in chapter 3. In addition to xylem tissue, roots and leaves (from nodes 4, 8, and 15) were also harvested from these plants and total stem tissue up to node 8 was extracted from plants grown in parallel for sulphate and thiol analysis. Sulphate levels were found to be higher in pathogen-inoculated roots, xylem and total stem tissue than in corresponding control material at 7 dpi. This increase also occurred in leaves 4 and 8, but later at 14 dpi and later still in leaf 15 increasing at both 14 and 21 dpi. Glutathione content of xylem tissue and leaves from *V. dahliae*-challenged resistant but not susceptible or control plants increased approximately 2 to 3 fold at 14 dpi but no significant increase was detected in total stem tissue or root samples. Cysteine levels followed a similar pattern and increased approximately 2 to 3 fold at 14 dpi but only in the resistant xylem tissue challenged with *V. dahliae* (Williams et al., 2002; appendix 3 for data).

The tomato plants used to analyse changes in gene expression during *V. dahliae* infection were grown, inoculated and the xylem harvested from the lower stem identically to those used in the sulphate and thiol experiments described above, and the elemental sulphur analysis described in chapter 3. Therefore these experiments may be compared directly.

There was constitutive expression in leaf tissue of control plants of all sulphur metabolism genes as expected (M. Hawkesford pers. comm.) and so this tissue was used as a positive control in Northern analysis. The expression of the sulphate transporter, APS reductase and glutathione synthetase increased steadily during the time course in control xylem. This increase may perhaps be expected as the plant ages, as it may require more sulphate to sustain the increased biomass. It may also be a result of growth of the plant in a confined amount of soil for 11 weeks despite regular feeding with a high sulphate fertiliser.

By sequence alignment LeST2 is classed as a group 1 sulphate transporter which in general have a high affinity for sulphate, are primarily expressed in plant roots and are believed to be involved in sulphate uptake from the soil (Grossman and Takahashi, 2001; Yoshimoto et al., 2002). However LeST2 is expressed in the xylem tissue of the roots, stems and leaves (J. Howarth and M. Hawkesford, unpublished data) and so may also be involved in the internal distribution of sulphate within the plant, a role generally thought to be performed by group 2 sulphate transporters. The more intensive up-regulation of this vascular sulphate transporter gene at 7 dpi in the *V. dahliae*-inoculated xylem tissue compared to control xylem may be related to the higher level of sulphate found by S. Hall in challenged xylem tissue of the stem at 7 dpi (Williams et al., 2002; appendix A3.1). This occurred prior to the production of elemental sulphur that began and rapidly accumulated in this tissue between 8 and 14 dpi and then continued to accumulate up to 21 dpi (chapter 3). The up-regulation of this sulphate transporter may also have played a role in the accumulation of sulphate that was detected in the higher leaves (leaves 4, 8 and 15) at 14 dpi (Williams et al,. 2002; appendix A3.1). To the author's knowledge no previous work has so far been carried out specifically on the expression of sulphate transporters in response to pathogen challenge. However sulphate transporters that are expressed in the roots of plants and believed to be involved in sulphate uptake from the soil, are rapidly up-regulated in response to sulphur starvation (Smith et al., 1995a; Smith et al., 1997; Takahashi et al., 1997; Bolchi et al., 1999; Vidmar et al., 1999; Takahashi et al., 2000; Vidmar et al., 2000). Although the expression of vascular sulphate transporters in response to sulphur starvation has not been studied so extensively, increased expression of a vascular transporter has been detected in *Arabidopsis* roots and leaves during sulphate deficiency (Takahashi et al., 2000). It may be that pathogen-inoculation places a burden on sulphur metabolism in order to produce compounds involved in plants defence such as glutathione (Gullner and Kömives, 2001), thionins (Florack and Stiekema, 1994) defensins (Broekaert et al., 1995), and elemental sulphur. This burden may cause sulphur deficiency within the plant and subsequent up-regulation of the whole sulphur metabolism pathway. Therefore increased sulphate levels and increased expression of the sulphate transporters would be expected to occur before a significant accumulation of elemental sulphur.

Coincident with the sulphate transporter, APS reductase was also more intensively up-regulated at 7 dpi in the *V. dahliae*-challenged xylem in comparison to the control tissue, again suggesting an increased demand on sulphur metabolism in response to the pathogen. Again this up-regulation occurred prior to the accumulation of elemental sulphur, which may rely on increased APS reductase expression in order to occur. Increased APS reductase expression has previously been shown to occur during sulphate starvation (Gutierrez-Marcos et al., 1996; Takahashi et al., 1997) and more importantly during conditions that increase demand for glutathione and cysteine, including oxidative stress (Leustek et al., 2000). Oxidative stress is known to occur during plant pathogen interactions linked with the oxidative burst and has been implicated in plant defence by triggering and/or executing the HR (chapter 1). APS reductase is thought to be a key regulator of the sulphate reduction pathway (Leustek et al., 2000) and may therefore provide a major link between pathogen attack and up-regulation of the sulphur metabolism pathway in order to produce elemental sulphur.

The expression of both the cytosolic and plastidic cysteine synthase genes was high in the xylem of the lower stem during normal plant metabolism as shown by the band at 0 dpi in control and pathogen-inoculated tissue. In control xylem, the expression of both cysteine synthase isoforms was decreased at 7 dpi, perhaps in response to the root wounding given to the plants prior to pathogen inoculation. Their expression was then increased again between 14 and 21 dpi. However in *V. dahliae*-inoculated xylem, cysteine synthase expression remained high at 7 and 14 dpi for the plastidic form and perhaps was even up-regulated at 7 dpi for the cytosolic form in comparison to control plants. These results again can be compared to those of S. Hall where cysteine levels increased approximately 2 to 3 fold from 7 to 14 dpi, after the peak in sulphate accumulation, suggesting that cysteine production is enhanced in response to pathogen challenge from reduction of sulphate (Williams et al., 2002; appendix A3.2). Cysteine synthase is not down-regulated, as occurred in controls, perhaps due to the requirement for products of sulphur metabolism in defence. This requirement for sulphur may again be enhanced by the need to accumulate elemental sulphur. In support of this data, up-regulation of cysteine synthase has previously been detected in various plants in response to sulphate starvation and heavy metal exposure. (Hell et al., 1994; Barroso et al., 1995; Barraso et al., 1999; Hesse et al., 1999; Nakamura et al., 1999). Increased specific activity of cysteine synthase during sulphur starvation

has also been observed (Passera and Ghisi, 1982; Hesse et al., 1999). Heavy metal exposure may require increased levels of glutathione in order to produce the phytochelatins that sequester the heavy metals and to detoxify ROS that are often generated during this type of stress (Cobbett, 2001; Hall, 2002) thereby placing a burden on cysteine synthase and the whole of sulphur metabolism. Elemental sulphur production may place a similar burden on sulphur metabolism. Cysteine is known to regulate negatively sulphate transporters and so any process that requires additional cysteine would deplete internal cysteine and encourage enhanced sulphate uptake (Herschbach and Rennenberg, 1991; Lappartient and Touraine, 1996; Smith et al., 1997; Bolchi et al., 1999). In plants undergoing sulphur deficiency, OAS is thought to accumulate due to a lack of sulphide to form cysteine (Kim et al., 1999). OAS is suggested to act as a positive regulator of sulphate transporter expression and the sulphur metabolism pathway, in order to provide sulphide for production of cysteine (Neuenschwander et al., 1991; Smith et al., 1997). OAS may therefore be involved in regulating the production of sulphide required for this cysteine synthase in response to pathogen attack. SAT, the enzyme used to produce OAS, has also been found to be up-regulated in response to sulphur starvation (Takahashi et al., 1997) and it would be interesting to see if this enzyme is also up–regulated in response to pathogen challenge.

Like the sulphate transporter, APS reductase and cysteine synthase genes, there is a more intensive increase in expression of glutathione synthetase at 7 dpi in *V. dahliae*-inoculated xylem in comparison to control xylem. Results by S. Hall showed an increase of approximately 2 to 3 fold in total glutathione at 14 dpi in xylem, leaf 4, leaf 8 and leaf 15 that may be related to the increase in glutathione synthetase expression at 7 dpi (Williams et al., 2002; appendix A3.3). It is known that glutathione and cysteine synthesis are integrated, as adding cysteine enhances glutathione production in many plants and the same seems to occur here with a peak of cysteine and glutathione occurring in the xylem concurrently. (Farago and Brunold, 1994; Schneider and Bergmann, 1995; Strohm et al., 1995; Noctor et al., 1996; Williams et al., 2002). Induction of glutathione synthetase has not previously been examined in response to sulphur deficiency, but like cysteine synthase a moderate increase has been found during stress induced by heavy metal exposure (Schäfer et al., 1998; Xiang and Oliver, 1998). Glutathione, like cysteine, also negatively regulates sulphate transporters and so any process that uses either of these compounds such as heavy metal exposure or perhaps

elemental sulphur production would encourage up-regulation of sulphate transporters as seen in this study (Herschbach and Rennenberg, 1991; Herschbach and Rennenberg, 1994; Lappartient and Touraine, 1996; Smith et al., 1997; Bolchi et al., 1999; Takahashi et al., 2000). Experiments have also shown that expression of γ-glutamylcysteine synthetase is also increased under heavy metal stress far more than that of glutathione synthetase (Schäfer et al., 1998; Xiang and Oliver, 1998). γ-glutamylcysteine synthetase is believed to have a major role in the regulation of glutathione synthesis and so it is justifiable to monitor expression of this gene in response to pathogen attack.

The enhanced expression of the sulphate transporter, APR reductase, cysteine synthases and glutathione synthetase appears to be short lived as it occurs only in response to pathogen challenge at 7 dpi. The expression of these genes is similar in control and pathogen-inoculated xylem at later time points suggesting that this burst of up-regulation in sulphur metabolism enhances the pools of sulphate, cysteine and glutathione sufficient to provide all of the sulphur-containing products required for defence, including presumably elemental sulphur. Furthermore, following the peak of sulphate at 7 dpi and cysteine and glutathione at 14 dpi, there was a subsequent decline in these pools to control levels at later time points (Williams et al., 2002; appendix 3) suggesting that they are sequentially metabolised into other components that also may be defence-related. Significant accumulation of elemental sulphur occurs at 14 dpi and then increases further up to 21 dpi (chapter 3). It is therefore possible that any of these components, sulphate, cysteine and glutathione, could be involved in elemental sulphur production. In order to confirm the above up-regulation of sulphur metabolism genes the Northern analysis will need to be repeated. Northern analyses carried out by J. Howarth with the sulphate transporter and APS reductase have also shown up-regulation in response to the pathogen, but at all time points (J. Howarth pers. comm.). It would also be interesting to look at the other genes involved in sulphur metabolism, particularly SAT and γ-glutamylcysteine synthetase and genes of methionine biosynthesis to give a more complete picture of how sulphur metabolism is modified; however only γ-glutamylcysteine synthetase has so far been cloned from tomato (Kovari et al., 1997).

An attempt was made by J. Howarth to isolate genes differentially expressed in resistant tomato plants in response to *V. dahliae* by creating a subtractive cDNA

library. This technique yielded many false positives. Only one clone appeared to be up-regulated following reverse Northern analysis. This was designated G7-3 and contained a short domain showing homology to rhodanese genes (sulphurtransferases). Sulphurtransferases comprise a group of enzymes widely distributed in plants, animals and bacteria that catalyse the transfer of a sulphane atom from a donor molecule to a thiophilic acceptor substrate. Rhodanese, the best characterised sulphurtransferase catalyses the reaction between thiosulphate and cyanide to form thiocyanate and sulphite (Papenbrock and Schmidt, 2000). The role of this enzyme in plants is poorly understood and the idea that it may play a role in assimilatory sulphate reduction by transferring a molecule of sulphide to OAS has not been excluded (Schmidt and Jäger, 1992). Therefore this gene was analysed by Northern analysis both at the University of Bath and by J. Howarth at IACR Rothamsted. However G7-3 was also found to be a false positive as negligible differences in expression between the control and pathogen-inoculated xylem were detected over the time course. Furthermore, the full-length rhodanese clone also showed no changes in expression in response to the pathogen.

cDNA-AFLP analysis performed by J. Howarth also yielded many false positives. However one clone, designated 6-5, was up-regulated specifically in response to *V. dahliae* at all time points and in all parts of the plant. This has no homology to any other genes and is now subject to rapid amplification of cDNA ends (RACE) cloning of the 3' and 5' ends to give a full length sequence with which to determine its function *in vivo*.

As yet it is impossible to form a model from the above data but it is possible to put forward a theory as to how elemental sulphur may be produced (Fig. 4.4). In prokaryotes elemental sulphur is thought to originate from the oxidation of sulphide. How this oxidation occurs is a controversial issue. For some elemental sulphur producing bacteria, for example *Chromatium vinosum*, *Chlorobium limicola* f. *thiosulfatophilum* and *Beggiatoa alba,* sulphide oxidation has been suggested to occur by the action of flavocytochrome *c* with a variety of small, soluble *c*-type cytochromes as electron acceptors (Bartsch and Kamen, 1960; Meyer et al., 1968; Kusai and Yamanaka, 1973; Fukumori and Yamanaka, 1979; Gray and Knaff, 1982; Schmidt et al., 1987; Cusanovich et al., 1991). However this enzyme is absent in some sulphide-oxidising bacteria (Meyer et al., 1973; Fischer and Trüper, 1979; Steinmetz et al., 1983; Wermter and Fischer, 1983; Meyer, 1985).

The transfer of electrons directly into the quinone pool by sulphide-quinone oxidoreductase has also been implicated in the oxidation of sulphide by *C. vinosum* and *C. limicola* f. *thiosulfatophilum* mentioned above as well as in *Oscillatoria limnetica*, *Rhodobacter capsulatus*, *Chlorobium tepidum* and *Paracoccus dentrificans* (Arieli et al., 1991; Shahak et al., 1992; Shahak et al., 1994; Klughammer et al., 1995; Schütz et al., 1997; Friedrich, 1998; Reinartz et al., 1998). Furthermore, a distinct enzyme, sulphide oxidase, has been implicated in the formation of elemental sulphur in *Thiobacillus ferrooxidans* and *Hyphomicrobium neptunium* (Sasahira et al., 1993; Bang et al., 1995). It is possible that sulphide oxidation may also occur in plants by one of these mechanisms, although as yet both sulphide-quinone oxidoreductase and sulphide oxidase have only been reported in prokaryotes.

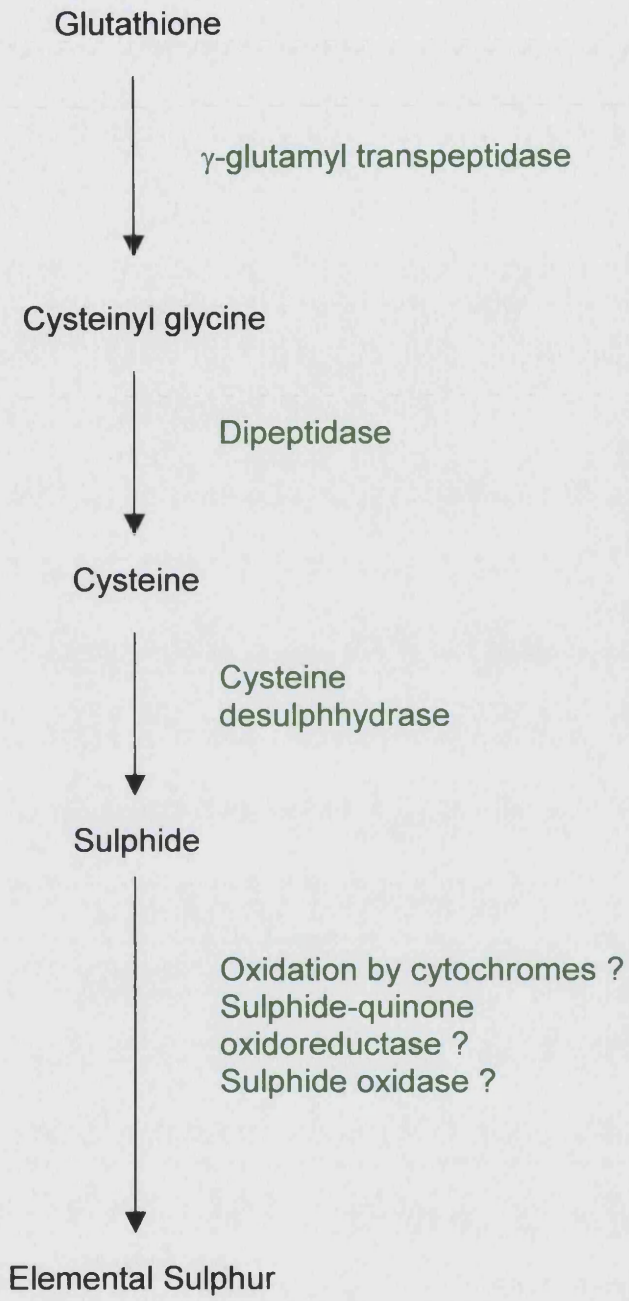

Figure 4.4 A proposed pathway for elemental sulphur production in plants. The intermediates and products of this pathway are shown in black and the enzymes involved in their production in green. All of these enzymes are known to exist in higher plants except sulphide oxidase and sulphide-quinone oxidoreductase that have only so far been found in prokaryotes. However none of these enzymes have as yet been cloned completely and only γ-glutamyl transpeptidase has been purified in a higher plant (Lancaster and Shaw, 1994; Martin and Slovin, 2000).

The source of the sulphide in plants may be from direct reduction of sulphate. However, given the up-regulation of genes involved in cysteine and glutathione synthesis seen here and the accumulation of their products detected by S. Hall, it is hypothesised that the sulphide may come from degradation of glutathione. As described previously, glutathione is thought to play a major role in plant defence against biotic and abiotic stresses including defence against pathogens where it is thought to play a role in detoxification of ROS and possibly elicitation of defence responses (De Kok and Stulen, 1993; Lamoureux and Rusness, 1993; Marrs, 1996; Foyer et al., 1997; Kömives et al., 1998; May et al., 1998; Noctor et al., 1998a; Edwards et al., 2000; Tausz and Grill, 2000; Cobbett, 2001; Gullner and Kömives, 2001; Hall, 2002). It is therefore a suitable candidate to be involved in elemental sulphur production. Perhaps the initial or primary role of glutathione is as an antioxidant and elemental sulphur production is a fortuitous spin off. The mobility of glutathione enables the long distance transport of reduced sulphur compounds and so it could be transported to the XP cells to form elemental sulphur, and its effect on sulphate uptake from the soil by negatively regulating sulphate transporters would allow changes in its pool size to influence sulphur metabolism (De Kok and Stulen, 1993; Brunold and Rennenberg, 1997).

The degradation of glutathione to cysteine is known to occur in plants but its role is poorly understood. There is also uncertainty as to the sequence of reactions for glutathione degradation in plants. Two pathways of glutathione degradation are thought to be possible (Bergmann and Rennenberg, 1993; Leustek et al., 2000). Glutathione may be hydrolysed to γ-glutamylcysteine by a carboxypeptidase and γ-glutamylcysteine may then be further degraded to cysteine by the successive actions of glutamylcyclotransferase and 5-oxo-prolinase. However as γ-glutamylcysteine is the substrate for glutathione synthesis and both are thought to take place in the cytoplasm this pathway seems unrealistic (Bergmann and Rennenberg, 1993). Perhaps a more feasible pathway is the same as that seen in animal cells in which glutathione is first hydrolysed to cysteinyl-glycine by γ-glutamyl transpeptidase and then further hydrolysed to cysteine by cysteinyl-glycine dipeptidase (Bergmann and Rennenberg, 1993). In contrast to glutamylcyclotransferase and 5-oxo-prolinase, that have only been detected in tobacco (Rennenberg et al., 1981; Steinkamp et al., 1987), γ-glutamyl transpeptidase activity has been detected in a wide range of plants including maize, *Arabidopsis* and tomato (Leustek et al., 2000). Furthermore the use of a γ-

glutamyl transpeptidase inhibitor inhibited glutathione degradation in tobacco (Schneider and Rennenberg, 1992). Therefore the second pathway is the preferred pathway of glutathione degradation. Until recently neither gene in this pathway had been cloned but a gene with homology to animal γ-glutamyl transpeptidase has now been isolated by screening an *Arabidopsis* cDNA library (Kushnir et al., 1995). Whether this gene product has γ-glutamyl transpeptidase activity remains to be seen.

Once cysteine has been formed, it may be degraded further by the action of a cysteine desulphhydrase to form sulphide. Cysteine desulphhydrase activity provides a major pathway for the degradation of sulphur-containing amino acids and has previously been reported in several plants including *Arabidopsis*, oil seed rape, cucurbit, tobacco and spinach (Rennenberg and Grundel, 1985; Schmidt, 1987; Schutz et al., 1991; Burandt et al., 2001; Burandt et al., 2002). The emission of hydrogen sulphide produced by this enzyme may directly play a role in plant defence, as it is highly toxic and may act as a fungicide (Sekiya et al., 1982; Beauchamp et al., 1984; Schröder, 1993). Hydrogen sulphide may also act as a sulphide source for oxidation to elemental sulphur. However as yet a specific cysteine desulphhydrase gene has not been cloned, nor has the enzyme been purified. Although a separate enzyme is thought to be involved in cysteine degradation to sulphide in plants as in yeast (Aida et al., 1969), it is now known that cysteine desulphhydrase activity is also a side reaction of cysteine synthase and this is thought to function along side cysteine desulphhdrases (Burandt et al., 2002). Whether cysteine or sulphide is formed depends on pH and on the cellular compartment (Schmidt, 1982; Burandt et al., 2002). Cysteine desulphhydrase activity is assayable (Schmidt, 1987; Burandt et al., 2001) and it would therefore be interesting to see if its activity is increased in resistant tomato plants in response to *V. dahliae*.

Chapter 5

Toxicity of Elemental Sulphur to Bacterial and Fungal Plant and Non Plant Pathogens

5.1 Introduction

Elemental sulphur is probably the oldest of all pesticides. As far back as 1000 BC Homer, the Greek epic poet spoke of "the pest-averting sulphur with its properties of divine and purifying fumigation" (Horsfall, 1956; Sharvelle, 1961). Forsyth (1802), first recommended sulphur for the control of diseases and this was followed by Robertson (1824), who reported that sulphur was the only remedy that he could find for the treatment of peach mildew. Soon after elemental sulphur became widely recognised as a valuable fungicide for the control of mildews on fruit and vegetable crops, scab on apples and pears, and leaf curl in peaches (Horsfall, 1956; Sharvelle, 1961; Tweedy, 1981; Paul and Rossignol, 1982). To this day sulphur still insures the protection of most vineyards against several parasites (Jolivet, 1993) and protects many other economically important crops such as cashew trees and pea against mildew (Smith et al., 1995b; Warkentin et al., 1996). Its negligible toxicity to animals and low toxicity to plants have made sulphur attractive as a chemical control agent and as a component of integrated pest management programmes because of its low toxicity to beneficial insects (Markosyan, 1970). Elemental sulphur is a non-systemic fungicide and repeated applications may be required to give good protection (McGrath and Johnston, 1986). Despite this elemental sulphur has never been totally replaced by newer synthetic compounds such as carbendazim, maneb, benzimidazoles and demethylation inhibitory (DMI) fungicides and it is often applied in combination with them (Paul and Rossignol, 1982; Falisse and Bodson, 1987; Galet, 1996). In the light of increased resistance to many mildew fungicides (Koller and Scheinpflug, 1987; McGrath, 1996; Ypema et al., 1997) the mixing of elemental sulphur with these other fungicides may increase their lifespan as they use different sites of action (Jolivet, 1993; Galet, 1996).

The literature on the toxicity of elemental sulphur to various pathogens is many decades old and can be very confusing due to the different chemical formulations of sulphur used in the investigations i.e. sulphur flour (ground elemental sulphur), sulphur flowers (sublimed elemental sulphur), milk of sulphur (precipitated

elemental sulphur), colloidal sulphur (wettable form of any of the previous forms), liver of sulphur (potash containing elemental sulphur) and lime-sulphur (sulphide of lime containing elemental sulphur) (Thatcher and Streeter, 1925; Sharvelle, 1961). Furthermore many different bioassays have been used to assess toxicity to different plant pathogens. Some of these bioassays are *in vitro* laboratory based experiments to determine spore germination or spore viability on exposure to elemental sulphur (Foreman, 1910; Doran, 1922; Young, 1922; McCallan and Wilcoxon, 1931; Wilcoxon and McCallan, 1931; Miller et al., 1953; Owens, 1960; Tweedy and Turner, 1966; Beffa, 1993b) and others are *in vivo* laboratory, greenhouse or field based experiments where plant material is assessed for infection or symptoms after treatment with sulphur (Pierce, 1900; Eyre and Salmon, 1916; Uppal and Malelu, 1928; Martin, 1930; Martin and Salmon, 1932; Jones, 1934). These bioassays have often given very different or conflicting results, for example in some studies *Botrytis cinerea* was considered to be completely resistant to elemental sulphur (Foreman, 1910; Barker et al., 1920; Marsh, 1929) and in others this fungus was considered highly susceptible (Doran, 1922). The main aim of most of these investigations was to determine the mode of action of the toxicity of elemental sulphur to pathogens, rather than the level of toxicity, which also remains unresolved (Tweedy, 1981; Jolivet, 1993; Beffa, 1993b).

In order to implicate elemental sulphur production in the resistance of plants to vascular pathogens (chapter 3), the levels of sulphur required to inhibit both vascular fungi (*Verticillium dahliae*, *Fusarium oxysporum*) and bacteria (*Ralstonia solanacearum*, *Erwinia stewartii*) were investigated. Due to the inconsistencies in the literature, it was also decided to survey a range of fungal and bacterial plant pathogens from different fungal classes for elemental sulphur toxicity, including the pathogen for which elemental sulphur is famous for controlling, powdery mildew. Also included in this survey were a range of *Verticillium* and *Fusarium* spp. and isolates, some of which are destructive vascular pathogens, some of which are weak vascular pathogens and some are non-vascular pathogens. This was to determine if there were any differences in elemental sulphur susceptibility between pathogens that colonise the xylem of plants successfully (and may therefore be exposed to elemental sulphur) and those that cannot. Also included in the survey were Gram-negative plant pathogenic bacteria and a Gram-positive non plant pathogenic bacterium (*Bacillus cereus*), as it has previously been observed that

Gram-positive bacteria are generally more sensitive than Gram-negative bacteria to phytoalexins and antibiotics (Smith and Banks, 1986). To determine the toxicity of elemental sulphur to these pathogens, pure, ground ^{32}S (Sigma, Poole, UK) was used and several laboratory-based bioassays were developed. For all fungi except *Ustilago maydis*, bioassays were developed to test toxicity to spore germination, mycelial growth or both. For *U. maydis,* which grows in a yeast-like budding form and for bacteria, multiplication in the presence of elemental sulphur was determined.

5.2 Materials and Methods

5.2.1 Source, growth and maintenance of pathogens to be tested for susceptibility to elemental sulphur and the diseases that they cause

Source and details of pathogens tested for sensitivity to elemental sulphur

The name, isolate, disease caused and source for each of the pathogens tested for sensitivity to elemental sulphur are described in Tables 5.1, 5.2, 5.3 and 5.4 below.

Pathogen	Isolate	Source
V. albo-atrum	43 ex tomato	F. Veazey (University of Bath Culture Collection, Bath, UK)
V. albo-atrum	Str3 ex lucerne	D. Barbara (HRI, Wellesbourne, UK)
V. dahliae	Dvd T5 Race 1 ex tomato	K. Dobinson (Agriculture and Agri-food, Ontario, Canada)
V. dahliae	Col 1 ex cocoa	F. Veazey (University of Bath culture collection, Bath, UK)
V. dahliae	1200B ex strawberry	A. Soares (HRI, East Malling, UK)
V. dahliae	Australian field isolate ex cotton	S. Allen (Australian Cotton Research Institute, Narrabri, NSW, Australia)
V. longisporum	VdIV ex oil seed rape	A. Collins (HRI, Wellesbourne, UK)
V. longisporum	ex horseradish	D. Barbara (HRI, Wellesbourne, UK)
V. nubilum	162 ex tomato	D. Barbara (HRI, Wellesbourne, UK)
V. tricorpus	JKG 20b ex linden (*Tilia*)	J. Goud (Wageningen Agricultural University, Netherlands)
F. oxysporum f. sp. *nicotianae*	ex tobacco	J. LaMondia (Connecticut Agricultural Experiment Station, Connecticut, USA)
F. oxysporum f. sp. *lycopersici*	33 ex tomato	F. Veazey (University of Bath Culture Collection, Bath, UK)
F. oxysporum f. sp. *phaseoli*	B13 ex bean	H. Schwartz (Colorado State University, Colorado, USA)
F. oxysporum f. sp. *vasinfectum*	24596 01111 ex cotton	N. Moore (Farming Systems Institute, Indooroopilly, Queensland, Australia)

Table 5.1 Source of *Verticillium* and *Fusarium* fungal vascular pathogens of plants. Note, all of the above fungi are classed as Anamorphic or Imperfect fungi (as they have no known perfect or sexual stage) (Agrios, 1997; Kirk et al., 2001) and cause a wilt disease on the host. *V. dahliae*, *V. albo-atrum* and *V. longisporum* are classed as strong plant pathogens causing serious losses in a wide variety of major crops (Pegg, 1974; Heale, 1988; Heale, 2000). *V. tricorpus* and *V. nubilum* are classed as weak pathogens causing mild symptoms on host plants (Isaac, 1967; Korolev and Gindin, 1999).

Pathogen	Isolate	Disease Caused	Family	Source
Verticillium theobromae	225818 ex banana	Cigar-end rot	Anamorphic/ Imperfect	J. Flood (CABI Bioscience, Egham, UK)
Fusarium oxysporum f. sp. *radicis-lycopersici*	Ashford ex tomato	Foot and root rot	Anamorphic/ Imperfect	J. Carder (HRI Wellesbourne, UK)
Fusarium oxysporum f. sp. *narcissi*	GCRI-X ex daffodil	Basal and neck rot	Anamorphic/ Imperfect	J. Carder (HRI Wellesbourne, UK)
Fusarium oxysporum f. sp. *tulipae*	CBS 242.59 ex tulip	Bulb rot	Anamorphic/ Imperfect	J. Carder (HRI Wellesbourne, UK)
Fusarium avenaceum	F17 ex potato	Root rot	Anamorphic/ Imperfect	A. Klees (SCRI, Dundee, Scotland)
Colletotrichum lindemuthianum	Isolate 1020 race 2047	Anthracnose	Anamorphic/ Imperfect	G. Roca (Federal University of Lavras, Lavras, Brazil)
Cladosporium fulvum	CF4 ex tomato	Leaf mould	Anamorphic/ Imperfect	P. Spanu (Imperial College, London, UK)
Stagonospora nodorum	BS171 ex barley	Leaf and glume blotch	Anamorphic/ Imperfect	L. Bindschedler (University of Bath, Bath, UK)
Monilinia fructigena	ex apple	Brown rot	Ascomycete	F. Veazey (University of Bath Culture Collection, Bath, UK)
Erysiphe graminis	Glasshouse isolate Maintained on barley	Powdery mildew	Ascomycete	Maintained on barley (University of Bath, Bath, UK)
Phytophthora palmivora	Tri1 ex cocoa	Black pod, stem canker and chupon wilt	Oomycete	M. Ducamp, (CIRAD, Montpellier, France)
Ustilago maydis	AB ex maize	Maize smut	Basidiomycete	F. Veazey (University of Bath Culture Collection, Bath, UK)

Table 5.2 Source of fungal non-vascular pathogens of plants.

Pathogen	Isolate	Disease Caused	Source
Ralstonia solanacearum (Gram –ve)	GMI 1000 ex tomato	Bacterial wilt	C. Boucher (INRA, Toulouse, France)
Pseudomonas syringae pv. *tomato* (Gram –ve)	DC3000 ex tomato	Bacterial speck	J. Mansfield (Imperial College at Wye, Ashford, UK)

Table 5.3 Source of bacterial pathogens of plants.

Pathogen	Isolate	Disease Caused	Source
Bacillus cereus (Gram +ve)	ID27	Human food poisoning	F. Veazey (University of Bath Culture Collection, Bath, UK)
Verticillium fungicola var. *fungicola*	7-4 ex Agaricus	Mushroom dry bubble	D. Barbara (HRI, Wellesbourne, UK)
Verticillium lecanii	V115-74 ex *Scolytus scolytus* (large elm bark beetle)	Insect parasite	F. Veazey (University of Bath Culture Collection, Bath, UK)
Verticillium chlamydosporium	77 ex nematode	Parasite of cyst and root-knot nematode eggs and sedentary females	D. Barbara (HRI, Wellesbourne, UK)

Table 5.4 Source of fungal and bacterial non plant pathogens.

Growth and maintenance of pathogens

All *Fusarium* and *Verticillium* spp., *C. fulvum*, *S. nodorum* and *C. lindemuthianum* were stored long term as spore suspensions in glycerol (25% v/v) at -70°C. When required 10 µL of the spore suspension was pipetted onto the appropriate agar medium and incubated to produce a spreading radial culture. The bacteria *R. solanacearum*, *P. syringae*, *B. cereus,* and basidiospores of the fungus *U. maydis* were also stored long term in glycerol (25% v/v) at –70°C and streaked out onto the appropriate medium and incubated to produce individual colonies when required. *P. palmivora* was stored long term as agar plugs of mycelium in distilled water (pH 6.5) at room temperature and *M. fructigena* was stored on agar slants under liquid paraffin at -4°C. Colonised agar was transferred to the appropriate agar medium and incubated when required. *E. graminis* is an obligate parasite and cannot be cultured on artificial nutrient media. It was therefore maintained on barley plants in the glasshouse. Successive inoculations were made onto spring barley (cultivar Golden Promise) by brushing spores from infected leaves onto healthy plants. The media and growth conditions for each pathogen are described in Tables 5.5, 5.6 and 5.7 below.

Pathogen	Growth Medium	Incubation temperature	Incubation time	References
V. albo-atrum (tomato isolate)	Czapek dox agar or potato dextrose agar (PDA)	22-24°C	10-14 d	Schnathorst (1981)
V. albo-atrum (lucerne isolate)	Czapek dox agar or PDA	24-26°C	10-14 d	Christen et al. (1983)
V. dahliae (all isolates)	Czapek dox agar or PDA	24-26°C	10-14 d	Puhalla and Bell (1981)
V. longisporum (all isolates)	Czapek dox agar or PDA	24-26°C	10-14 d	Karapapa et al. (1997)
V. nubilum	Czapek dox agar or PDA	24-26°C	10-14 d	Griffiths (1982)
V. tricorpus	Czapek dox agar or PDA	24-26°C	10-14 d	Huisman (1988); Li et al. (1994)
V. theobromae	Czapek dox agar or PDA	24-26°C	10-14 d	J. Flood pers. comm. (CABI Bioscience, Egham, UK)
V. fungicola	Czapek dox agar or PDA	24-26°C	10-14 d	Marlowe (1982)
V. lecanii	Czapek dox agar or PDA	24-26°C	10-14 d	Korolev and Gindin (1999)
V. chlamydosporium	Czapek dox agar or PDA	24-26°C	10-14 d	Dackman and Bååth (1989); Arora et al. (1996)
Fusarium spp.	Czapek dox agar or PDA	24-26°C	4-5 d	Booth (1971)

Details of the media constituents are given in appendix A1.1.

Table 5.5 Media and growth conditions for *Verticillium* and *Fusarium* spp.

Pathogen	Growth Medium	Incubation temperature	Incubation time	References
C. fulvum	PDA	23-25°C	10-14 d	Spanu (1997)
S. nodorum	Czapek dox V8 complete supplement (CzV8CS) agar	18-20°C	6-8 d	Newton and Caten (1988)
M. fructigena	PDA	21-22°C	10-14 d	Van Leeuwen and Van Kesteren (1998)
C. lindemuthianum	PDA	20-21°C	10 d	Roca (1997)
P. palmivora	V8 agar plus $CaCO_3$	24-26°C	7 d	Grant et al. (1985)
U. maydis	Czapek dox PLUS agar	28-30°C	3 d	Holliday (1974); Steinberg et al. (2000)
E. graminis	Barley plants (cultivar Golden Promise)	20-22°C	14 d	Hall et al. (1999)

Details of the media constituents are given in appendix A1.1.

Table 5.6 Media and growth conditions for fungal species other than *Verticillium* and *Fusarium*.

Pathogen	Growth Medium	Incubation temperature	Incubation time	References
R. solanacearum	BGT agar	28-30°C	2 d	Boucher et al. (1985)
P. syringae pv. *tomato*	NYGA	24-26°C	2 d	Turner et al. (1984); Li et al. (1998)
B. cereus	Nutrient Agar	28-20°C	2 d	Behravan et al. (2000); Charni et al. (2000)

Details of the media constituents are given in appendix A1.2.

Table 5.7 Media and growth conditions for bacterial pathogens.

5.2.2 Growth of pathogens in preparation for bioassays

5.2.2.1 Production of spores from *Verticillium* and *Fusarium* spp.

Spore suspensions of *V. dahliae*, *V. albo-atrum*, *V. nubilum*, *V. tricorpus*, *V. theobromae*, *V. lecanii*, and all *F. oxysporum* formae speciales were produced in shake cultures in Czapek dox liquid medium at 150 rpm in an orbital shaker (Gallenkamp, Loughborough, UK) with the required temperature for growth (Table 5.5). Four d, 20 mL starter cultures were produced from agar cultures in 50 mL centrifuge tubes and used to inoculate 100 mL cultures in 250 mL conical flasks that were incubated for a further 2d. Spore suspensions of *V. longisporum* isolates, *V. fungicola* and *V. chlamydosporium* were produced in the same way but with the addition of 0.1% yeast to the growth medium (A. Collins pers. comm.), and for *V. chlamydosporium* a 3d incubation in the 100 mL culture was given. Spore suspensions of *F. avenaceum* were produced from a 5d 100 mL shake culture grown in Czapek dox directly from an agar culture. Shake culturing induces the production of a mixture of spores, both blastospores from yeast-like growth and conidia (Goettel and Inglis, 1997). A pure conidial suspension was also produced from *V. dahliae* isolate Dvd T5. For this an agar culture was grown for 10d at 25°C and 10 mL of sterile distilled water (pH 6.5) pipetted into the Petri dish. The conidia were scraped off into the water using a sterile spatula. All spore suspensions were filtered through muslin to remove mycelial fragments.

5.2.2.2 Production of spores from other fungal pathogens

Ascomycetes and Anamorphs

Conidia production by *M. fructigena*, and *C. lindemuthianum* was achieved on agar cultures with the medium, temperature and incubation time as described above (Table 5.6) but with defined light conditions to aid or induce sporulation (Roca, 1997; Van Leewen and Van Kesteren, 1998). Both fungi were supplied with 12h light and 12h dark although for *M. fructigena* near UV light was supplied by 8W bulbs (Philips Electronics, London, UK) and for *C. lindemuthianum* fluorescent light was supplied by 8W white bulbs (Philips Electronics, London, UK). 10 mL of sterile distilled water (pH 6.5) was then pipetted onto the culture and the conidia dislodged into the water by scraping the culture with a sterile spatula. For *M. fructigena* only, the conidial suspension was whirled on a rotary shaker (IKA-VIBRAX-VXR, IKA Works Inc, Wilmington, North Carolina, USA) for 2 min at 2200 rpm to break up the conidial chains (Van Leewen and Van Kesteren, 1998). The

spore suspensions of both fungi were filtered through muslin to remove mycelial fragments.

Conidial suspensions of *C. fulvum* and *S. nodorum* were achieved by first producing spore lawns on agar. For *C. fulvum* a spore lawn was produced from spores present on agar cultures grown as explained previously (Table 5.6). Spores were dislodged from these colonies again with a sterile spatula into 3 mL sterile distilled water (pH 6.5), and 1 mL of this suspension spread onto fresh medium and incubated as described above (Table 5.6) in darkness to produce the spore lawn (P. Spanu pers. comm.). For *S. nodorum* spores were first induced on agar cultures by the addition of continuous near-UV light to the growth conditions described previously (Table 5.6) (Newton and Caten, 1988). A spore lawn was then produced as for *C. fulvum*. Spores present on spore lawns were dislodged from the cultures as above, suspended in 10 mL sterile distilled water (pH 6.5) and filtered through muslin to remove mycelial fragments.

E. graminis conidia were produced on barley plants grown in the glasshouse with a 16h photoperiod and were harvested directly from the plant when required. To ensure all spores were of similar age, the leaves of infected barley plants were brushed 24h prior to use to remove all old conidia and to allow new conidia to be produced (Carver and Ingerson, 1987).

Oomycete

Zoospores were produced from *P. palmivora* by a method modified from that of Bircher and Hohl (1997). *P. palmivora* was subcultured onto plates of V8 agar plus $CaCO_3$ and placed firstly in darkness at 24°C for 5d, and then with 16h light (near UV) and 8h dark for 3d to produce sporangia. Sporangia were harvested by adding 10 mL of sterile distilled water (pH 6.5) to the Petri dish and scraping the colony with a sterile spatula. The spore suspension was then incubated at 4°C for 20 min and then for an additional 20 min at 25°C and 50 rpm on an orbital shaker to induce the emergence of zoospores. After filtration through Whatman 541 filter paper the presence of zoospores was confirmed at 100X magnification on a haemocytometer. The suspension of zoospores was then vortexed for 1 min to cause encystment and therefore to induce synchronous germination (Tokunaga and Bartnicki-Garcia, 1971). In the absence of a stimulus zoospores encyst and

germinate asynchronously over a period of time which may vary from 1h to several d (Griffith et al., 1988).

Basidiomycete

Colonies of *U. maydis* were lifted from agar cultures produced as described previously (Table 5.6) and transferred to 20 mL of Czapek dox PLUS liquid medium (appendix A1.1). This was incubated overnight at 150 rpm at the required growth temperature (Table 5.6) to produce a turbid suspension of basidiospores (Holliday, 1974).

5.2.2.3 Production of bacterial cultures

Single colonies of *P. syringae*, *R. solanacearum* and *B. cereus* were lifted from agar and transferred to 20 mL NYGB, B broth, or Nutrient broth (appendix A1.2) respectively and were incubated overnight at 150 rpm at the temperatures required for growth (Table 5.7) to produce a turbid suspension of cells.

5.2.3 Bioassays

Slide bioassay to assess fungal spore germination

The slide bioassay was modified from that of Resende et al. (1996). All fungal pathogens were tested by this bioassay with the exception of *U. maydis.* Spore suspensions were centrifuged at 3,000*g* for 10 min, the pellet resuspended in sterile distilled water (pH 6.5) and diluted to 3 x 10^5 spores/mL. A 2-fold dilution series of ^{32}S (Aldrich, Gillingham, UK) ranging from 0.78 to 100 μg/mL as well as 500, 1,000 and 8,000 μg/mL solutions were made up in HPLC grade dichloromethane (Fisher, Loughborough, UK). Fifty μL of one of these solutions or pure solvent was pipetted into each of three 10 mm wells of a teflon-lined diagnostic slide (Merck, Darmstadt, Germany). The contents of each well was evaporated and 40 μL of the fungal spore suspension (5.2.2) added. In the case of *E. graminis,* conidia do not germinate in aqueous media but do germinate dry on glass slides in the presence of high humidity (Carver and Ingerson, 1987). Therefore infected wheat leaves were brushed over the treated diagnostic slides until the required number of spores were present on each well. All slides were then transferred to individual Petri dishes containing moistened filter paper and incubated for 15h at the temperature required for growth (Tables 5.5 and 5.6). Immediately after incubation, 10 μL of 0.1% (w/v) aniline blue in lactophenol was pipetted into each well to stain spores and arrest further growth, except for *E.*

graminis where conidia were observed without staining. Spores were observed at 200X magnification and in all cases were considered to have germinated when germ tube length was longer than the spore diameter. Conidia of *E. graminis* produce two morphologically distinct germ tubes, firstly one or more small primary germ tubes are produced followed by an appressorial germ tube which then arrests on the slides as glass is non-inductive to appressorium formation. Therefore conidia were considered to have germinated if they had produced at least one germ tube. Percentage germination was calculated from 100 spores from each well. Mean germ tube growth for all fungi except *E. graminis* was calculated from 10 spores selected at random for each of the three replicate wells. An overall germ tube growth mean and SE was then calculated from the three well means to account for both tube to tube variation and well to well variation. Data were subject to probit analysis (Finney, 1964), the method commonly used to determine an ED_{50} or potency value from a toxicity bioassay.

TLC bioassay to assess fungal spore germination and mycelial growth

The TLC bioassay was adapted from that described in Cole (1994). All fungal pathogens were tested by this bioassay with the exception of *U. maydis* and *E. graminis*. Spore suspensions (5.2.2) were centrifuged at 3,000*g* for 10 min. Spores were resuspended in the liquid medium required for growth on TLC (Table 5.8) and diluted with this medium to 1 x 10^6 spores/mL. A 20 x 20 cm TLC aluminium sheet (silica gel 60 F_{254}, Merck) was pre run in dichloromethane and allowed to dry. Fifty μL of each of the ^{32}S solutions quoted above and pure solvent as a control was pipetted slowly onto the TLC plate and the dichloromethane allowed to evaporate resulting in approximately 25 mm diameter zones. The spore suspension was then sprayed evenly onto the plate with a 200 mL aerosol sprayer (Fisher). Plates were incubated in darkness in a sealed container with moistened filter paper to give 100% relative humidity at the required temperature for growth (Tables 5.5 and 5.6). Plates were analysed daily for 7d and then weekly until 40d to detect inhibition of spore germination initially and then mycelial growth, which would eventually colonise the majority of the silica gel and could subsequently invade initial zones of inhibition. The way in which fungal growth was visualised on the TLC plate varied depending on the fungus. All *V. dahliae* isolates except the cotton isolate produced pigmented, melanised microsclerotia providing a sufficiently dark background to visualise white areas where growth did not occur. *V. tricorpus* (green), *V. chlamydosporium* (yellow), *F. avenaceum* (pink), *F.*

oxysporum f. sp. *tulipae* (purple), *F. oxysporum* f. sp. *radicis-lycopersici* (purple), *C. lindemuthianum* (brown), *C. fulvum* (green) and *S. nodorum* (pink) were also pigmented and therefore visualised easily. For all other fungi where growth was hyaline and therefore difficult to see, mycelium was visualised by placing the TLC into a sealed chamber containing 1g of iodine crystals (Sigma, Poole, UK). The iodine vapour stained the organic fungal growth as yellow/brown (Waldi, 1965). Toxicity of the sulphur to the fungi was quantified by determining the point at which a complete zone of inhibition of fungal growth was observed over a zone of sulphur application.

Fungus	Medium in which spores suspended
V. dahliae (all isolates) *V. albo-atrum* (both isolates) *V. nubilum* *V. tricorpus* *V. theobromae* *V. lecanii* *F. oxysporum* (all formae speciales) *F. avenaceum* *C. fulvum* *V. chlamydosporium*	Czapek dox liquid medium
P. palmivora	50% Czapek dox 50% pea broth
V. longisporum (both isolates) *V. fungicola* *M. fructigena*	Czapek dox liquid medium plus 0.1% yeast
S. nodorum	CzV8CS liquid medium
C. lindemuthianum	Mathur's liquid medium

Details of the media constituents are given in appendix A1.1

Table 5.8 Media in which fungal spores were suspended prior to application to the elemental sulphur treated TLC plate.

Disc bioassay to assess fungal mycelial growth

The disc bioassay was adapted from that described in Cole (1994). All fungal pathogens were tested by this bioassay with the exception of *U. maydis* and *E. graminis*. Fifty μL of each of the ^{32}S sulphur solutions quoted previously was pipetted slowly onto each of three replicate sterile 13 mm antibiotic assay discs (Whatman, Maidstone, UK). A further three discs were impregnated with ten 50 μL applications of the 8000 μg/mL solution and the discs allowed to dry between each application. As controls, a set of three discs received no treatment and a second

set received 50 μL of pure dichloromethane. After the solvent had evaporated the discs were applied to the appropriate growth medium for the fungus under investigation, and pressure was applied until the discs were seen to absorb some of the moisture from the agar. For *Verticillium* and *Fusarium* spp. disc bioassays were first attempted on Czapek dox. However, if the fungus would not grow over the control discs on this medium PDA was used. In the centre of the plate, equidistant from each of the three discs, 10 μL of fungal spore suspension (5.2.2) was applied. Plates were then incubated at the required temperature for growth (Tables 5.5 and 5.6) in the dark. When the leading edge of the fungal colony between the discs had grown beyond the outer edge of the discs, plates were examined visually to assess the pathogen's ability to grow over the sulphur treated discs. Each plate and the corresponding sulphur concentration was assigned a score ranging from 0 to 5 with 0 representing no coverage of the discs and a score of 5 for total colonisation of the discs.

Shaken culture bioassay to assess fungal and bacterial multiplication

The shaken culture bioassay was adapted from the suspension tests described in Russell et al. (1992). All bacterial pathogens and basidiospores of the fungus *U. maydis* were tested by this bioassay. Overnight shake cultures of *P. syringae, R. solanacearum*, *B. cereus* and *U. maydis* were produced (5.2.2) and diluted to approximately 10^5 bacteria or spores/mL with the same liquid growth medium. For *P. syringae* a suspension of 1 x 10^5 bacteria/mL was used as determined by OD and comparison to an OD/viable count curve (3.2.4). For *R. solanacearum* and *B. cereus* suspensions of 2.56 x 10^5 and 2.55 x 10^4 were used respectively (concentrations were initially estimated to be 1 x 10^5 bacteria/mL using the *P. syringae* OD/viable count curve and then more accurately quantified with a viable count performed on the suspension used in the bioassay). For *U. maydis* a spore concentration of 1 x 10^5 spores/mL was used as determined by haemocytometer.

One mL of each sulphur solution was pipetted into each of three replicate, 2 mL sterile glass vials. As controls three vials were left untreated and a further three were filled with 1 mL of pure dichloromethane. The solvent was then evaporated off with a speed vac concentrator (Stratech Scientific, Luton, UK) and 1 mL of the bacterial or fungal suspension pipetted into the vials. Tubes were sealed and incubated overnight at the temperature required for growth (Tables 5.6 and 5.7) at 150 rpm. Following incubation a viable count was performed on each tube and the

mean number of bacterial cells present at each sulphur concentration calculated. Growth of *U. maydis* was assessed by performing spore counts with a haemocytometer on each tube and the mean number of spores present calculated for each sulphur concentration.

5.3 Results

5.3.1 Effect of elemental sulphur on spore germination: Slide bioassay

The slide bioassay was designed to investigate the inhibition of fungal spore germination by elemental sulphur. A 2-fold dilution series of ^{32}S ranging from 0.78 to 100 μg/mL was made up in dichloromethane as well as solutions of 500, 1000 and 8000 μg/mL. For the initial fungi tested, the most dilute sulphur concentration used was 1.56 μg/mL however some fungi were sensitive even at this concentration and therefore a 0.78 μg/mL solution was produced and used for all subsequent fungi and all bioassays.

Spores from all species of fungi tested were able to germinate well on the untreated diagnostic slides. Treating slides with pure dichloromethane had little or no effect on this germination. However, elemental sulphur was highly inhibitory to the germination of all fungal spores with the exception of encysted zoospores of *P. palmivora* where both spore germination and germ tube growth were unaffected at all concentrations of sulphur (Figs. 5.1 to 5.14).

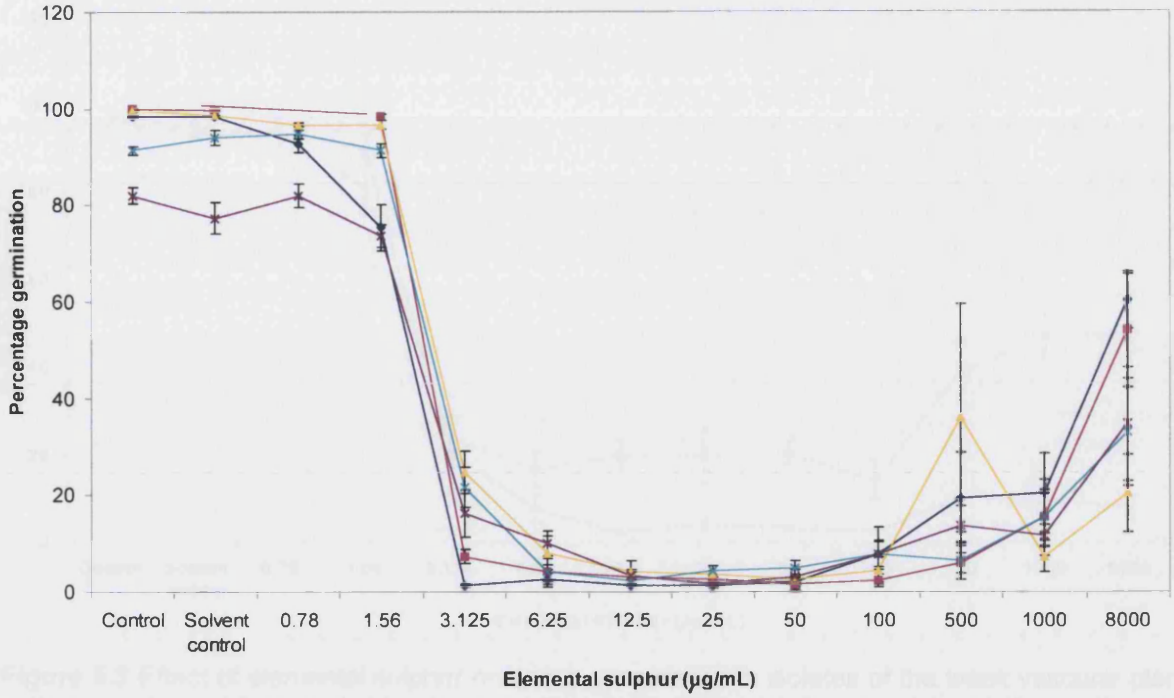

Figure 5.1 Effect of elemental sulphur on spore germination in isolates of the strong vascular plant pathogen *V. dahliae*. Both a suspension of pure conidia (×) and a mixed suspension of conidia and blastospores (■) of the *V. dahliae* tomato isolate were exposed to varying amounts of elemental sulphur. Mixed spore suspensions from cotton (◆), cocoa (▲) and strawberry (×) *V. dahliae* isolates were also tested. Values represent the percentage germination of three replicate analyses of 100 spores at each sulphur concentration with SE.

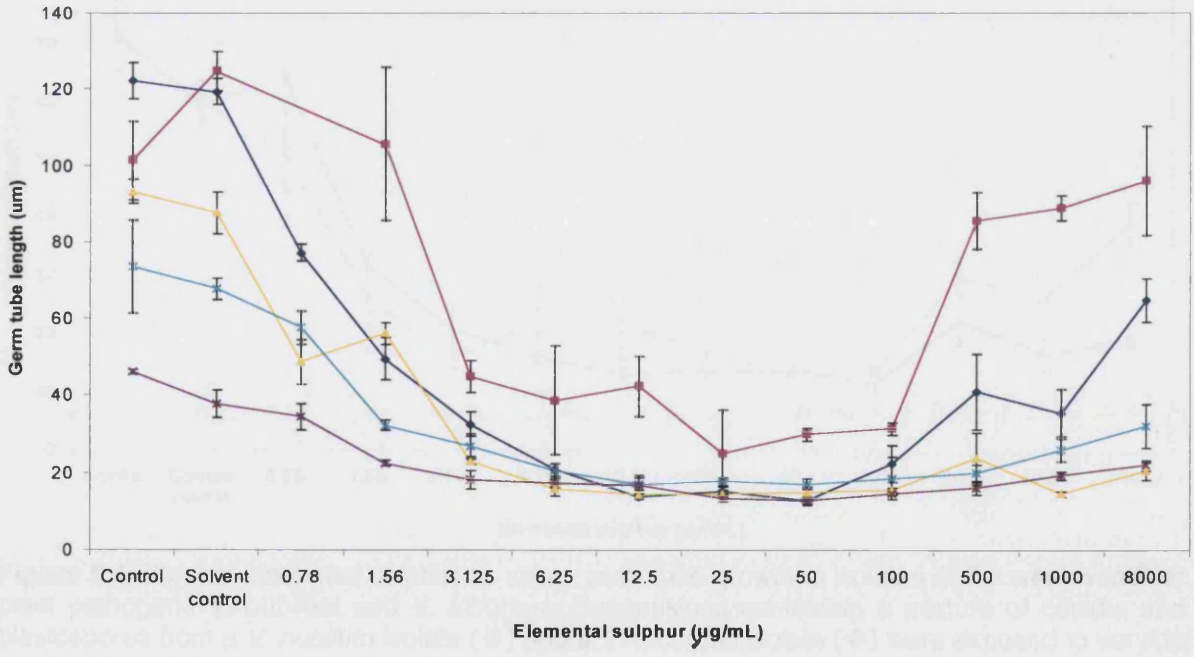

Figure 5.2 Effect of elemental sulphur on spore germ tube growth in isolates of the strong vascular plant pathogen *V. dahliae*. Both a suspension of pure conidia (×) and a mixed suspension of conidia and blastospores (■) of the *V. dahliae* tomato isolate were exposed to varying amounts of elemental sulphur. Mixed spore suspensions from cotton (◆), cocoa (▲) and strawberry (×) *V. dahliae* isolates were also tested. Values represent the mean germ tube length (µm) of 10 randomly selected germinating spores from each of three replicate analyses at each sulphur concentration with SE.

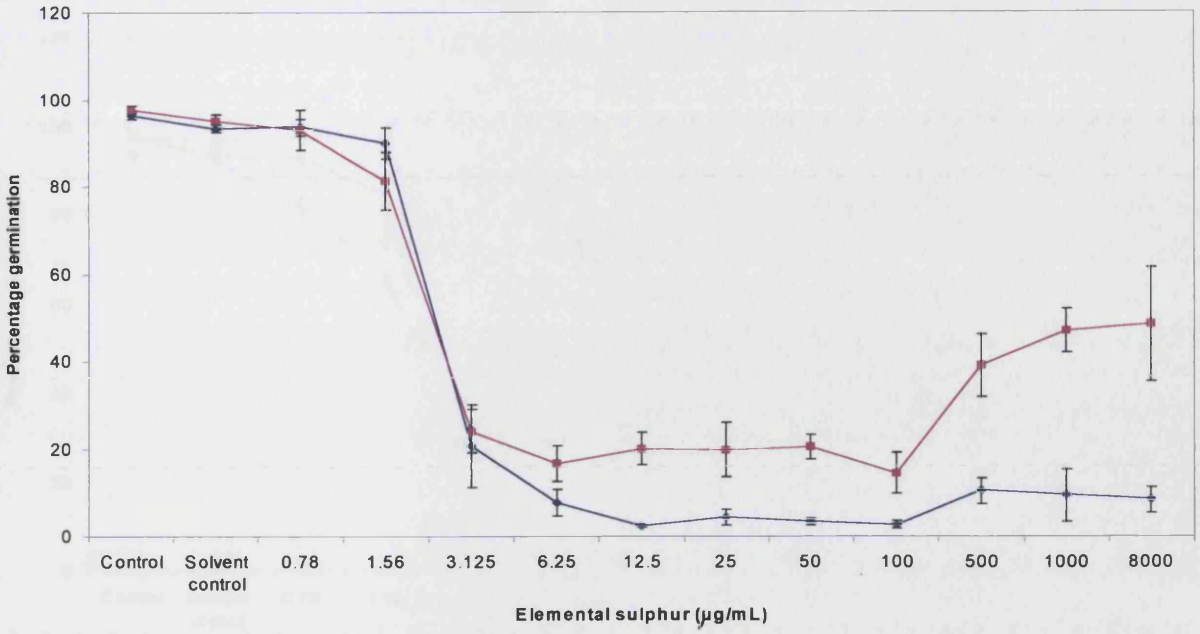

Figure 5.3 Effect of elemental sulphur on spore germination in isolates of the weak vascular plant pathogens *V. nubilum* and *V. tricorpus*. Suspensions containing a mixture of conidia and blastospores from a *V. nubilum* isolate (■) and a *V. tricorpus* isolate (◆) were exposed to varying amounts of elemental sulphur. Values represent the percentage germination of three replicate analyses of 100 spores at each sulphur concentration with SE.

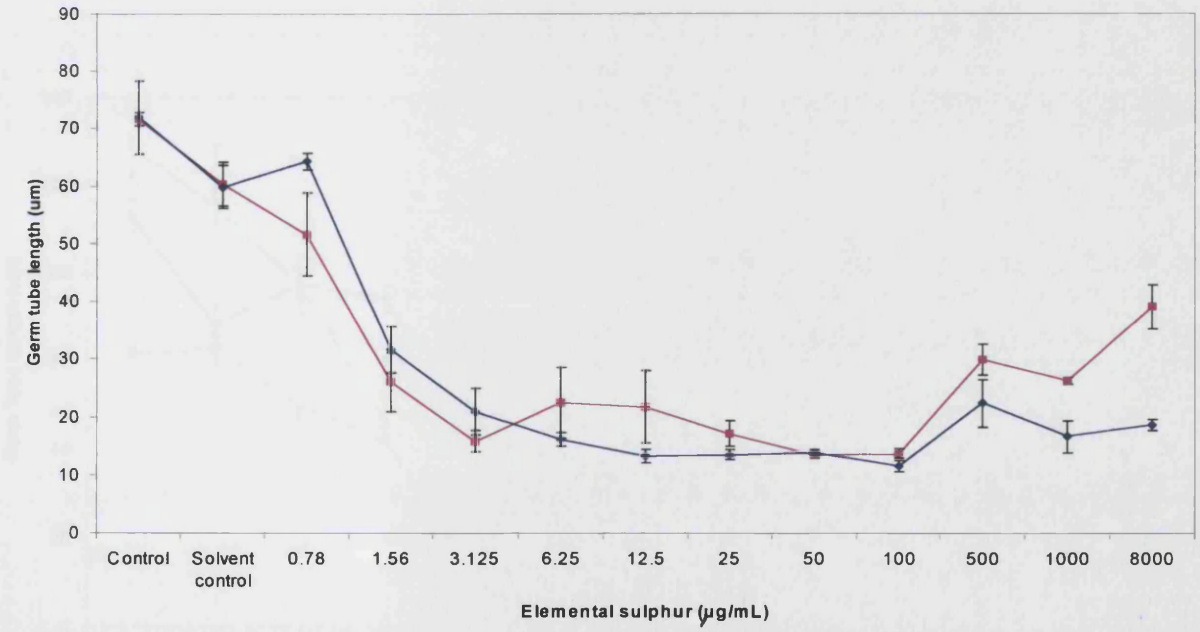

Figure 5.4 Effect of elemental sulphur on spore germ tube growth in isolates of the weak vascular plant pathogens *V. nubilum* and *V. tricorpus*. Suspensions containing a mixture of conidia and blastospores from a *V. nubilum* isolate (■) and a *V. tricorpus* isolate (◆) were exposed to varying amounts of elemental sulphur. Values represent the mean germ tube length (µm) of 10 randomly selected germinating spores from each of three replicate analyses at each sulphur concentration with SE.

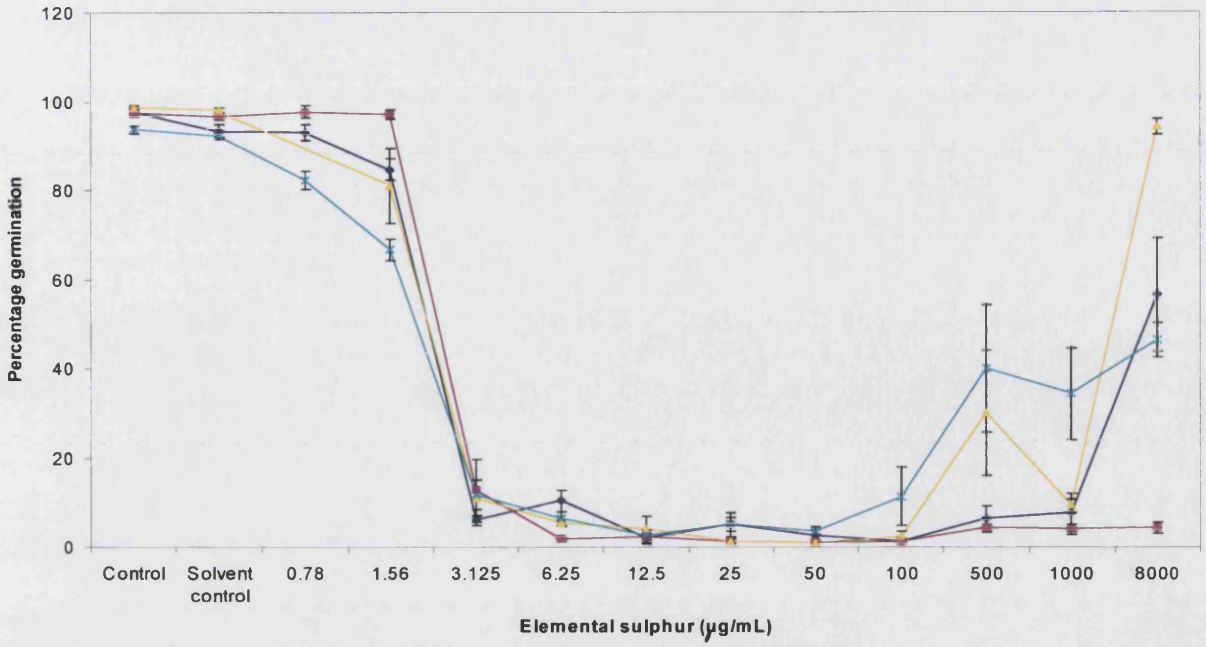

Figure 5.5 Effect of elemental sulphur on spore germination in isolates of the strong vascular plant pathogens *V. longisporum* and *V. albo-atrum*. Suspensions containing a mixture of conidia and blastospores from *V. longisporum* isolates from horseradish (■) and oil seed rape (◆) and *V. albo-atrum* isolates from tomato (▲) and lucerne (×) were exposed to varying amounts of elemental sulphur. Values represent the percentage germination of three replicate analyses of 100 spores at each sulphur concentration with SE.

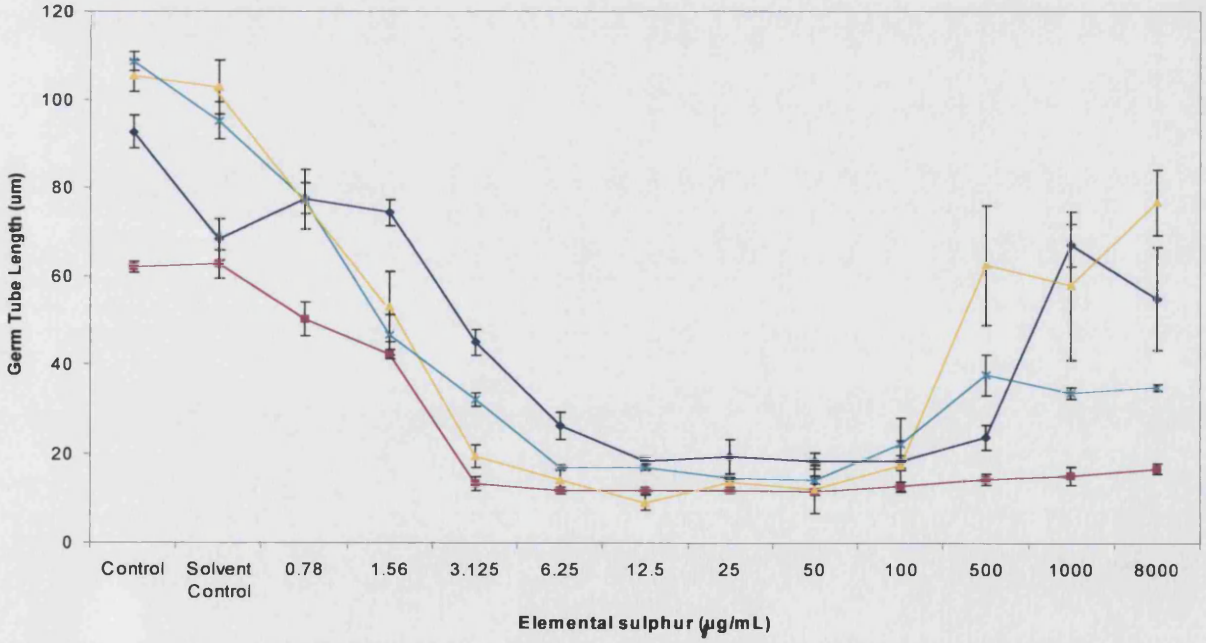

Figure 5.6 Effect of elemental sulphur on spore germ tube growth in isolates of the strong vascular plant pathogens *V. longisporum* and *V. albo-atrum*. Suspensions containing a mixture of conidia and blastospores from *V. longisporum* isolates from horseradish (■) and oil seed rape (◆) and *V. albo-atrum* isolates from tomato (▲) and lucerne (×) were exposed to varying amounts of elemental sulphur. Values represent the mean germ tube length (µm) of 10 randomly selected germinating spores from each of three replicate analyses at each sulphur concentration with SE.

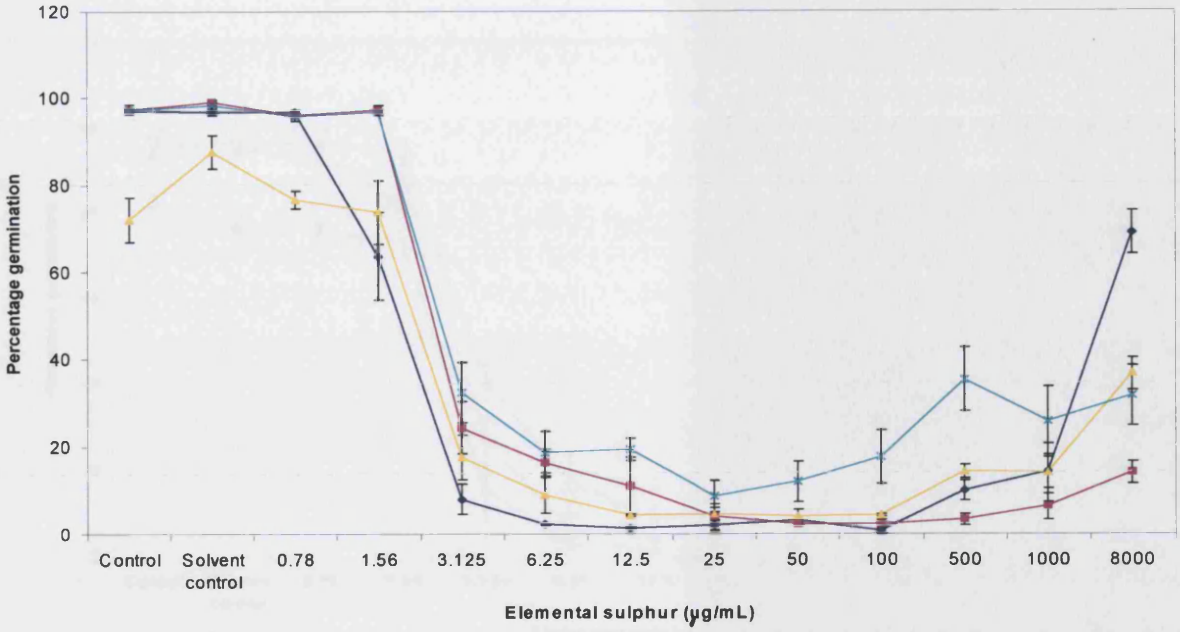

Figure 5.7 Effect of elemental sulphur on spore germination in formae speciales of the strong vascular plant pathogen *F. oxysporum*. Suspensions containing a mixture of conidia and blastospores from *F. oxysporum* f. sp. *vasinfectum* (■), *F. oxysporum* f. sp. *lycopersici* (◆), *F. oxysporum* f. sp. *phaseoli* (▲) and *F. oxysporum* f. sp. *nicotianae* (×) were exposed to varying amounts of elemental sulphur. Values represent the percentage germination of three replicate analyses of 100 spores at each sulphur concentration with SE.

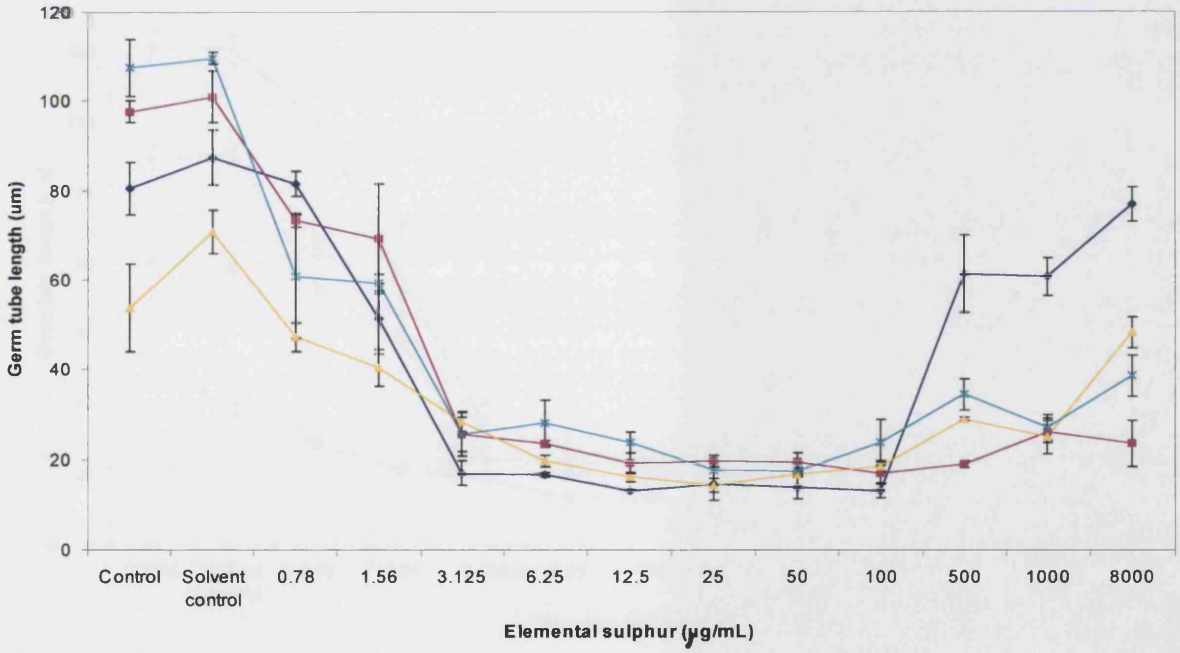

Figure 5.8 Effect of elemental sulphur on spore germ tube growth in formae speciales of the strong vascular plant pathogen *F. oxysporum*. Suspensions containing a mixture of conidia and blastospores from *F. oxysporum* f. sp. *vasinfectum* (■), *F. oxysporum* f. sp. *lycopersici* (◆), *F. oxysporum* f. sp. *phaseoli* (▲) and *F. oxysporum* f. sp. *nicotianae* (×) were exposed to varying amounts of elemental sulphur. Values represent the mean germ tube length (µm) of 10 randomly selected germinating spores from each of three replicate analyses at each sulphur concentration with SE.

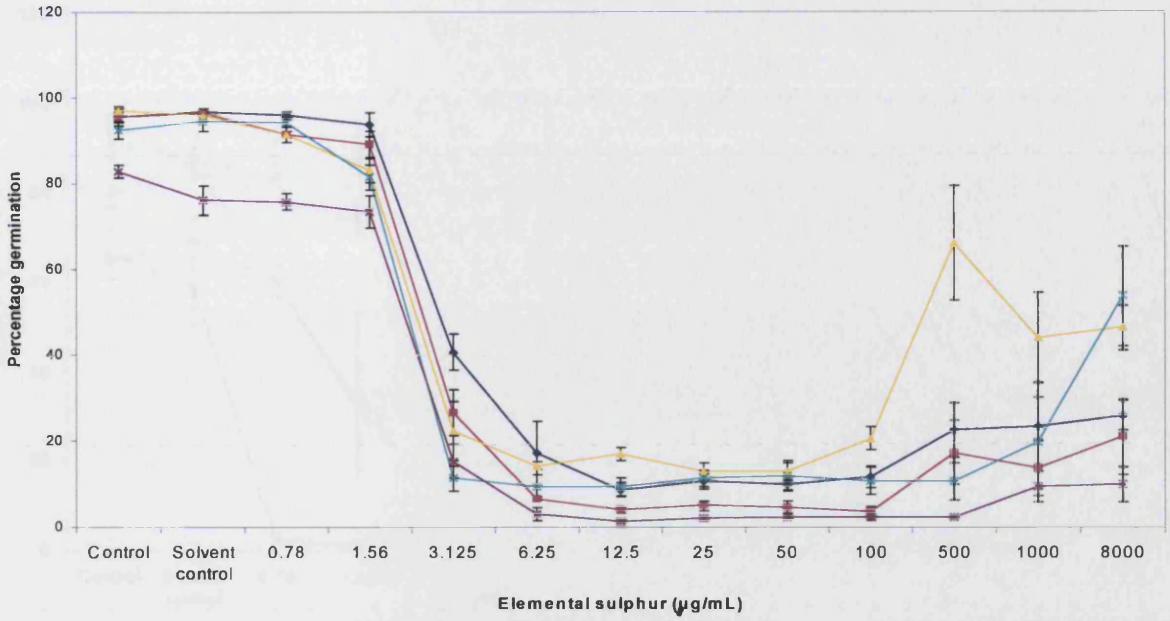

Figure 5.9 Effect of elemental sulphur on spore germination in isolates of *Fusarium* and *Verticillium* non-vascular plant pathogens. Suspensions containing a mixture of conidia and blastospores from *F. avenaceum* (■), *F. oxysporum* f. sp. *narcissi* (◆), *F. oxysporum* f. sp. *radicis-lycopersici* (▲), *F. oxysporum* f. sp. *tulipae* (×) and *V. theobromae* (X) were exposed to varying amounts of elemental sulphur. Values represent the percentage germination of three replicate analyses of 100 spores at each sulphur concentration with SE.

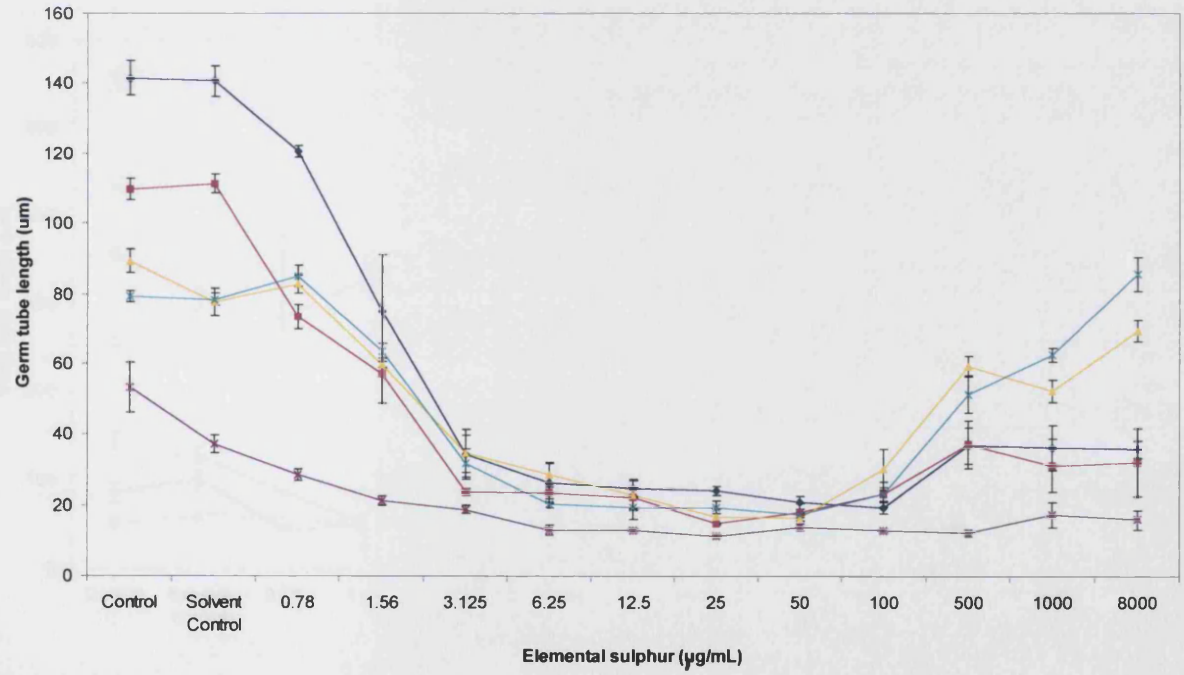

Figure 5.10 Effect of elemental sulphur on spore germ tube growth in isolates of *Fusarium* and *Verticillium* non-vascular plant pathogens. Suspensions containing a mixture of conidia and blastospores from *F. avenaceum* (■), *F. oxysporum* f. sp. *narcissi* (◆), *F. oxysporum* f. sp. *radicis-lycopersici* (▲), *F. oxysporum* f. sp. *tulipae* (×) and *V. theobromae* (X) were exposed to varying amounts of elemental sulphur. Values represent the mean germ tube length (µm) of 10 randomly selected germinating spores from each of three replicate analyses at each sulphur concentration with SE.

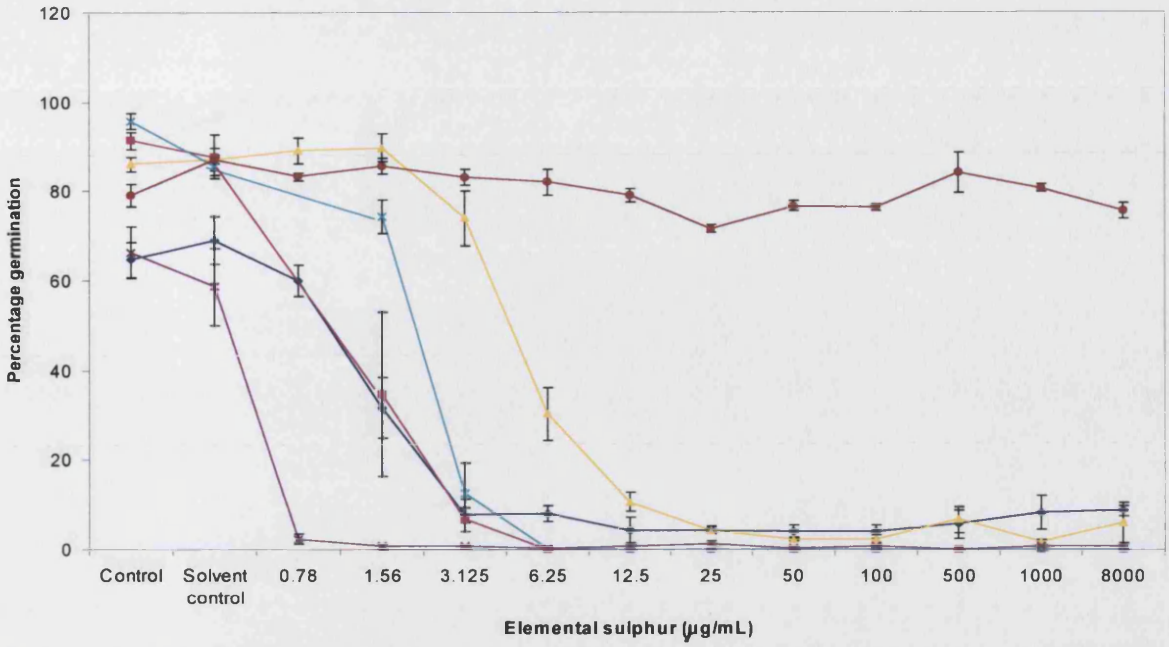

Figure 5.11 Effect of elemental sulphur on spore germination in isolates of non-vascular plant pathogens. Suspensions of conidia from *C. fulvum* (■), *C. lindemuthianum* (◆), *M. fructigena* (▲), *S. nodorum* (×), dry conidia from *E. graminis* (X) and suspensions of zoospores from *P. palmivora* (●) were exposed to varying amounts of elemental sulphur. Values represent the percentage germination of three replicate analyses of 100 spores at each sulphur concentration with SE.

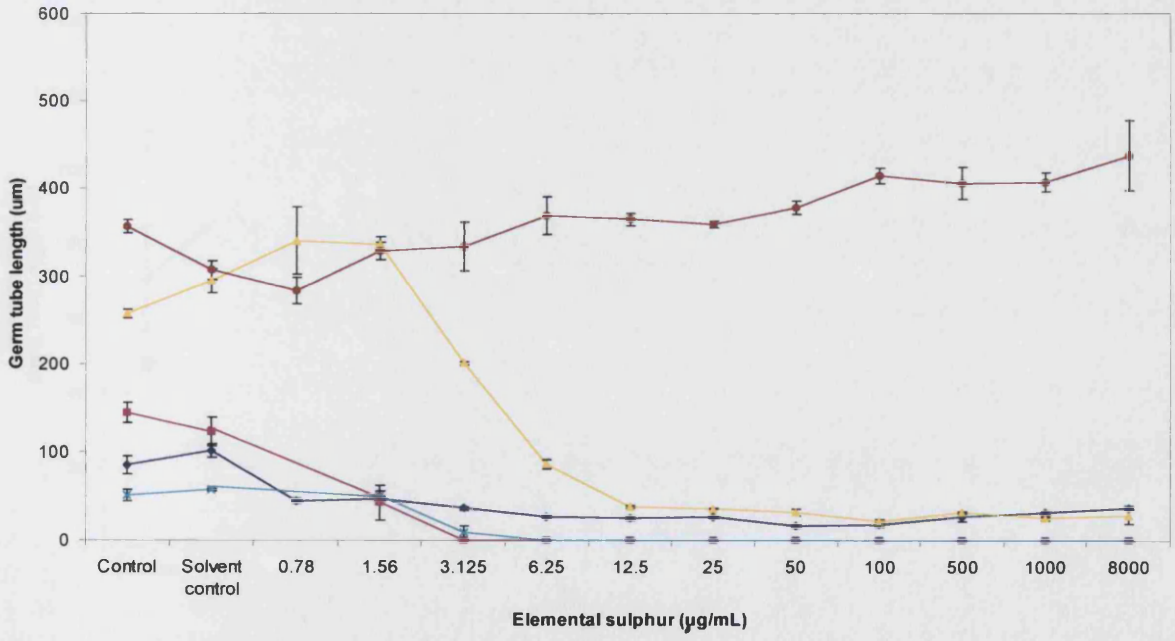

Figure 5.12 Effect of elemental sulphur on spore germ tube growth in isolates of non-vascular plant pathogens. Suspensions of conidia from *C. fulvum* (■), *C. lindemuthianum* (◆), *M. fructigena* (▲), *S. nodorum* (×) and suspensions of zoospores from *P. palmivora* (●) were exposed to varying amounts of elemental sulphur. Values represent the mean germ tube length (µm) of 10 randomly selected germinating spores from each of three replicate analyses at each sulphur concentration with SE.

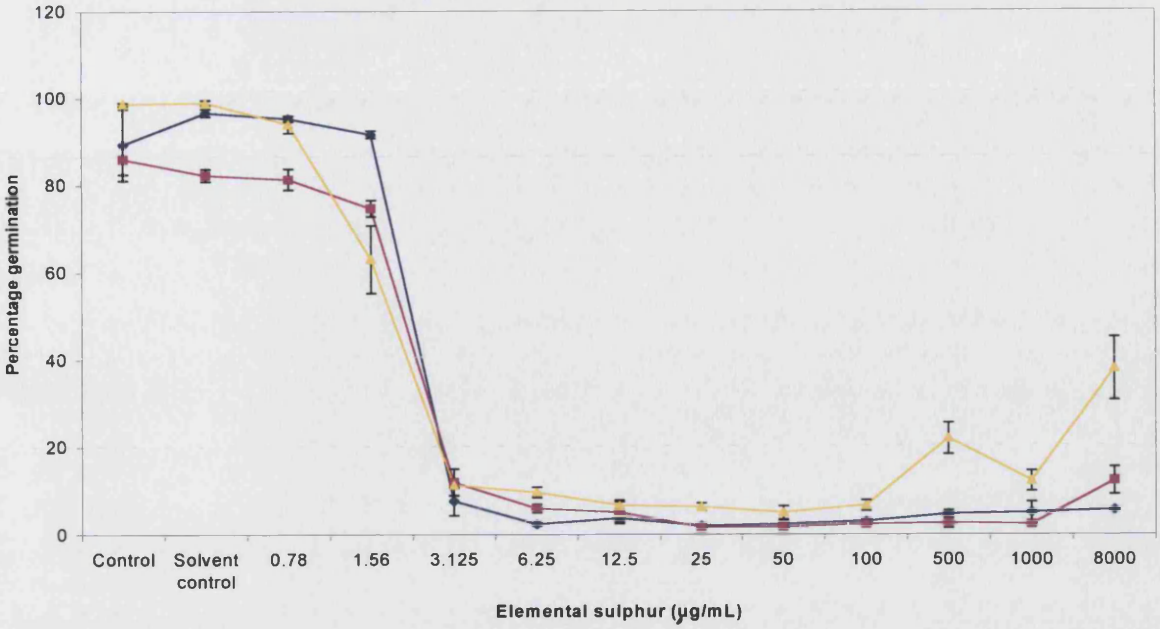

Figure 5.13 Effect of elemental sulphur on spore germination in isolates of non plant pathogenic *Verticillium* spp.. Suspensions containing a mixture of conidia and blastospores from *V. fungicola* (■), *V. lecanii* (◆) *and V. chlamydosporium* (▲) were exposed to varying amounts of elemental sulphur. Values represent the percentage germination of three replicate analyses of 100 spores at each sulphur concentration with SE.

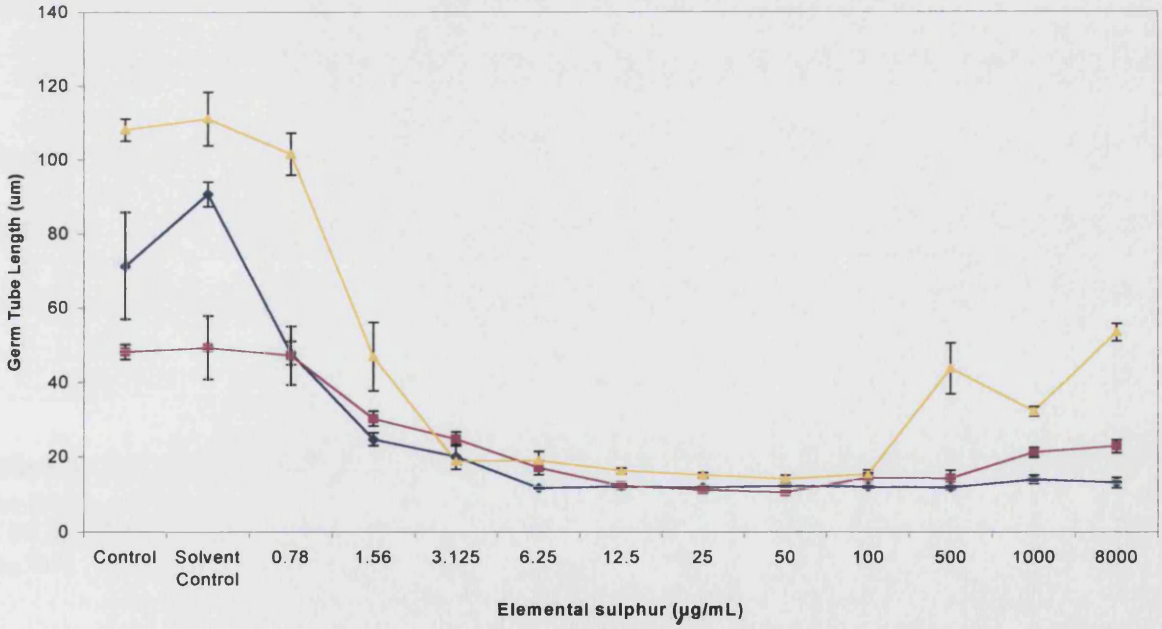

Figure 5.14 Effect of elemental sulphur on spore germ tube growth in isolates of non plant pathogenic *Verticillium* spp.. Suspensions containing a mixture of conidia and blastospores from *V. fungicola* (■), *V. lecanii* (◆) *and V. chlamydosporium* (▲) were exposed to varying amounts of elemental sulphur Values represent the mean germ tube length (µm) of 10 randomly selected germinating spores from each of three replicate analyses at each sulphur concentration with SE.

Probit analysis (the method commonly used to determine the potency of a toxin in a bioassay) was attempted on percentage germination data and germ tube growth data of elemental sulphur sensitive fungi and revealed that in most cases it did not fit a typical dose response. Data could therefore not be analysed to give an exact ED_{50} (the effective dose of a compound that reduces germination or germ tube growth of a fungus by 50%). Instead the ED_{50} is said to fall between two values as summarised in Table 5.9.

Pathogen	**ED_{50} germination value (μg/mL)**	**ED_{50} germ tube growth value (μg/mL)**
E. graminis	0 - 0.78	-
C. fulvum	0 - 1.56	0 -1.56
C. lindemuthianum	0.78 - 1.56	0 - 0.78
S. nodorum	1.56 - 3.125	1.56 - 3.125
Fusarium (all spp. and f. sp.)	1.56 - 3.125	1.56 - 3.125
V. dahliae (cotton isolate) *V. albo-atrum* (lucerne isolate) *V. nubilum* *V. lecanii* *V. chlamydosporium*	1.56 - 3.125	0.78 -1.56
V. longisporum (oil seed rape isolate) *V. theobromae* *V. fungicola*	1.56 - 3.125	3.125 - 6.25
V. dahliae (all isolates except cotton) *V. albo-atrum* (tomato isolate) *V. longisporum* (horseradish isolate) *V. tricorpus*	1.56 - 3.125	1.56 - 3.125
M. fructigena	3.125 – 6.25	3.125 - 6.25

Table 5.9 Toxicity of elemental sulphur to fungi as determined by ED_{50} percentage germination and ED_{50} germ tube growth. As the data was not suitable for probit analysis, an exact ED_{50} value could not be determined. The values given are the concentrations of elemental sulphur between which the ED_{50} fell.

The data were not obviously amenable to any other kind of statistical analysis making it difficult to compare fungi to determine whether they differed in susceptibility to elemental sulphur. Secondly, as the bioassays were not carried out all on the same day, which would be impracticable, it was difficult to determine the underlying variation that may occur for each fungus within the bioassay and

therefore again the fungi could not be statistically analysed for differential susceptibility (K. Ward pers. comm., Syngenta, Jealott's Hill International Research Centre, Bracknell, UK). However general trends could be noted.

Percentage germination data for all fungi sensitive to elemental sulphur gave a similar shaped response curve, a very rapid decline from a high percentage germination to almost complete inhibition of germination at a defined sulphur concentration (Figs. 5.1, 5.3, 5.5, 5.7, 5.9, 5.11, 5.13). All *Verticillium* and *Fusarium* spp. were very similar in their susceptibility to elemental sulphur regardless of their host or mode of disease with their ED_{50} values for percentage germination all falling within the same range of 1.56 - 3.125 μg/mL. The pure conidia of *V. dahliae* (tomato isolate) and the mix of blastospores and conidia from the same pathogen also showed no obvious differences in susceptibility to the sulphur. However differences were seen between different species of fungi. *E. graminis*, *C. lindemuthianum* and *C. fulvum* were shown to be more susceptible (ED_{50}) to elemental sulphur than *Verticillium* and *Fusarium* spp.. *S. nodorum* had an ED_{50} in the same range as *Fusarium* and *Verticillium* spp. and *M. fructigena* appeared more resistant (Table 5.9).

Germ tube growth data even in controls were highly variable between fungi in absolute values, which may be due to differences between fungi or small differences in the levels of nutrients in the spore suspensions used in the bioassays. For all sensitive fungi a reduction in germ tube growth was seen to accompany the decrease in percentage germination in response to the sulphur although it was a more gradual decrease than for percentage germination in most cases (Figs. 5.2, 5.4, 5.6, 5.8, 5.10, 5.12, 5.14). However the ED_{50} for germ tube growth fell within the same range as the ED_{50} for percentage germination for all fungi except *V. longisporum* (oil seed rape isolate), *V. theobromae* and *V. fungicola* where ED_{50} germ tube growth was one concentration range higher than the ED_{50} percentage germination; in contrast with *V. dahliae* (cotton isolate), *V. albo-atrum* (lucerne isolate), *V. nubilum*, *V. lecanii*, *V. chlamydosporium* and *C. lindemuthianum* where ED_{50} germ tube growth was one range lower than the ED_{50} percentage germination (Table 5.9).

Percentage germination and germ tube growth in all elemental sulphur sensitive fungi showed increasing inhibition until 100 μg/mL but in many cases inhibition was relaxed as the concentration of sulphur increased to 8000 μg/mL (Figs. 5.1 to 5.14). This appeared to be associated with the way in which the sulphur crystallised out of solution when the dichloromethane was evaporated from the treated wells of the diagnostic slides. If the concentration of sulphur applied in solution was low, the sulphur appeared to crystallise out as fine particles evenly distributed over the surface of the well. These particles appeared to be highly toxic to most fungal spores once they had reached a defined concentration on the slide. However when the concentration of sulphur applied was high (≥500 μg/mL), the elemental sulphur formed larger crystals that did not appear toxic to the fungi. In many cases spores appeared to be able to germinate directly on them (Fig. 5.15). At these higher concentrations it seemed that the percentage germination and germ tube growth were dependent on the amount of smaller sulphur particles present. In some cases there remained a layer of fine particles around the large crystals and therefore germination remained inhibited (*eg. V. longisporum* horseradish isolate). In other cases only larger crystals were present and the percentage germination and germ tube growth were increased dramatically (*eg. V. albo-atrum* tomato isolate). The amount of fine particles produced during crystallisation appeared random between bioassays but was possibly due to small changes in environmental conditions such as temperature between the different days on which the bioassays were carried out. Attempts were made to obtain repeatable crystallisation by evaporating dichloromethane on slides on heat blocks but variation was still found to occur.

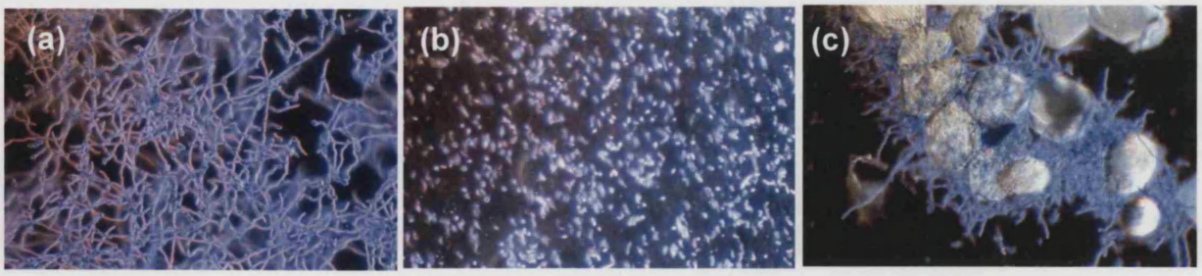

Figure 5.15 Germination of *V. dahliae* (tomato isolate) spores exposed to varying concentrations of elemental sulphur. Spores were observed at 200X magnification with phase contrast. Spores exposed to no sulphur showed a high percentage germination and long germ tubes (a). At 6.25 μg/mL of elemental sulphur, spores showed very low germination with an even layer of fine sulphur particles surrounding them (b) and at 8000 μg/mL of S^0, spores showed partially restored germination and germ tubes in the presence of large sulphur crystals (c).

5.3.2 Inhibition of spore germination and germ tube growth by elemental sulphur: TLC bioassay

The TLC bioassay was designed to investigate the toxicity of elemental sulphur to spore germination initially and then to mycelial growth resulting from the germination of spores in areas surrounding zones of sulphur application. All fungal pathogens were tested by this bioassay with the exception of *U. maydis* and *E. graminis*. Fungal growth on the TLC plate was visualised either by the natural pigmentation of the fungus or by exposing it to iodine vapour (Fig. 5.16).

All fungi were able to grow well on the untreated TLC plates in the medium supplied for growth (Table 5.8). Initially *V. longisporum*, *V. fungicola and M. fructigena* were suspended in Czapek dox medium prior to application but they did not grow on the TLC plate without the addition of 0.1% yeast.

All fungi except *P. palmivora* were shown to be susceptible to elemental sulphur by this method. Inhibition of growth was evident as white zones of uncolonised TLC plate against the pigmented or stained fungus (Fig. 5.16). Occasionally some fungi showed a slight inhibition of growth at a defined sulphur concentration, either as a ring of inhibition on the outer edge of the zone of application or as a slight thinning of growth over the zone of application (Fig. 5.16 a, b and d), however it was only at the point of complete inhibition of fungal growth over the application zone that the concentration of sulphur was noted as inhibitory. This excluded the appearance of small "micro colonies" of fungal growth, which often appeared even at very high sulphur concentrations within the complete inhibition zones (Fig. 5.16). These micro colonies were usually reduced in number as the concentration of sulphur increased but often some were still evident at 8000 μg/mL.

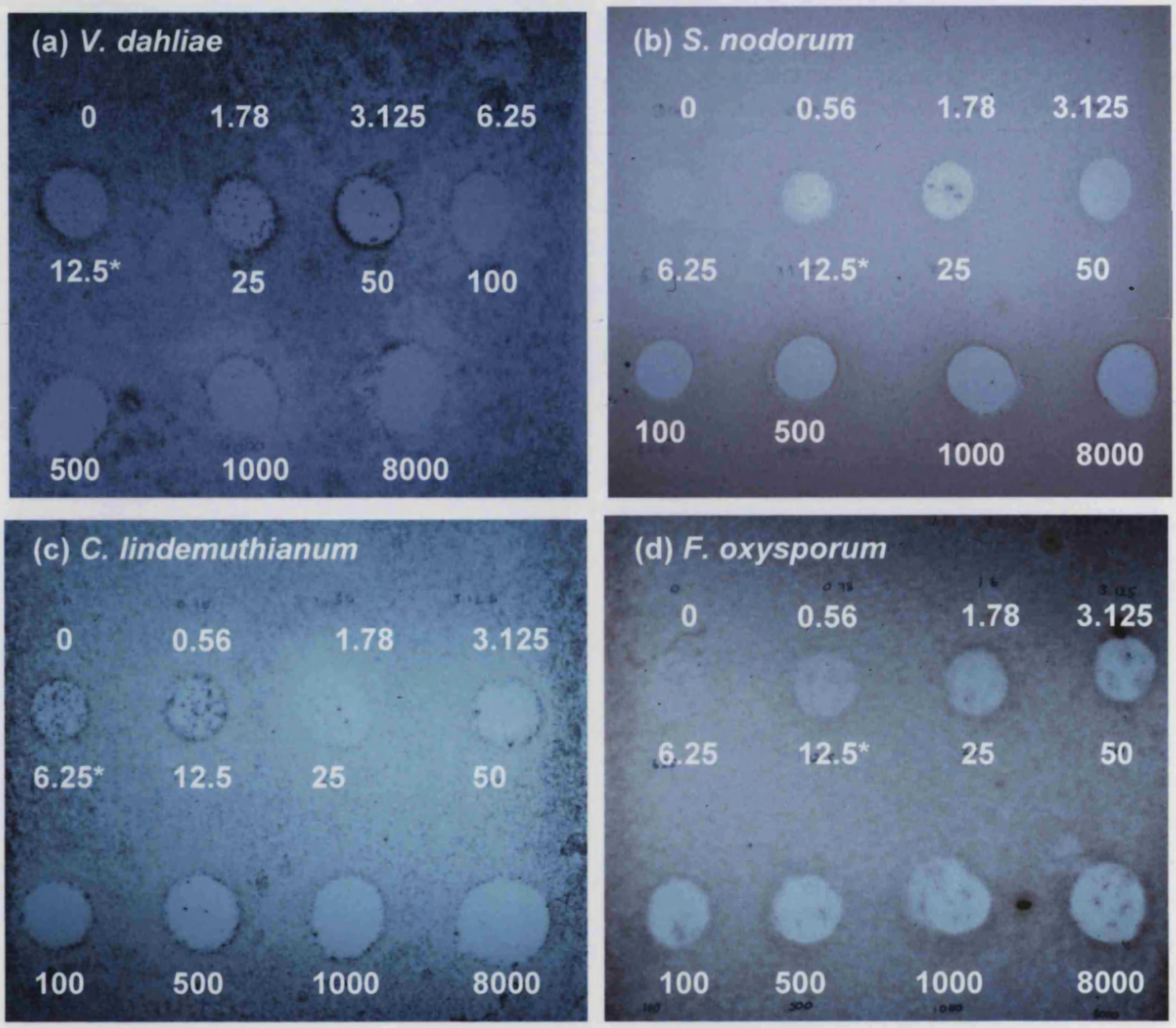

Figure 5.16 TLC bioassays showing toxicity of elemental sulphur to *V. dahliae* (tomato isolate), *S. nodorum, C. lindemuthianum* and *F. oxysporum* f. sp. *nicotianae* spores and mycelium. Fifty μL of each sulphur solution (concentration in μg/mL is shown below each application zone) was applied to the TLC plate and a suspension of the fungus (1 x 10^6 spores/mL) sprayed on the surface. *V. dahliae* was visualised as black pigmented microsclerotia, *S. nodorum* as pink pigmented mycelium, *C. lindemuthianum* as black pigmented conidia, and *F. oxysporum* f. sp. *nicotianae* was stained by exposure to iodine vapour. Toxicity of the sulphur to the fungi was quantified by determining the point at which a complete zone of inhibition of fungal growth was observed over a zone of sulphur application. Occasionally some fungi showed a slight inhibition of growth at a defined sulphur concentration, either as a ring of inhibition on the outer edge of the zone of application (3.125 and 6.25 μg/mL for *V. dahliae*) or as a slight thinning of growth over the zone of application (6.25 μg/mL for *S. nodorum* and *F. oxysporum*). However, it was only at the point of complete inhibition of fungal growth over the application zone that the concentration of sulphur was noted and marked with an asterisk. This excluded the appearance of small "micro colonies" of fungal growth, which often appeared even at very high sulphur concentrations within the complete inhibition zones (a, b, c and d). Definite zones of inhibition extending beyond the area of application at concentrations of 100 μg/mL to 8000 μg/mL can be noted for *V. dahliae* and *C. lindemuthianum* and dark halos surrounding inhibition zones can be seen for all fungi. This growth pattern did not change significantly even after 40d.

Fungus	Concentration of S^0 at which complete inhibition occurs (μg/mL)
F. oxysporum f. sp. *lycopersici* f. sp. *tulipae*	25
F. oxysporum f. sp. *nicotianae* f. sp. *phaseoli* f. sp. *vasinfectum* f. sp. *narcissi* f. sp. *radicis-lycopersici*	12.5
F. avenaceum	12.5
Verticillium (all spp. and isolates)	12.5
S. nodorum	12.5
M. fructigena	12.5
C. lindemuthianum	6.25
C. fulvum	6.25

Table 5.10 Inhibition of fungal growth on TLC plates impregnated with elemental sulphur. The values show the concentration of elemental sulphur required to inhibit completely fungal growth over the application zone. For any fungi where a value other than 12.5 μg/mL was obtained the experiment was repeated to confirm the result.

Species of *Verticillium* (including the pure conidia and the mixed suspension of conidia and blastospores from *V. dahliae* (tomato isolate)) were all very similar in their susceptibility to elemental sulphur as were *S. nodorum, M. fructigena*, *F. avenaceum* and most formae speciales of *F. oxysporum.* For all of these fungi complete inhibition occurred at 12.5 μg/mL. *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *tulipae* appeared more resistant with complete inhibition at 25 μg/mL. *C. lindemuthianum* and *C. fulvum* appeared more sensitive with complete inhibition at 6.25 μg/mL. For *P. palmivora* complete inhibition never occurred. The fungus grew over all concentrations of elemental sulphur.

For *V. dahliae* isolates, *C. lindemuthianum* and *C. fulvum* inhibition extended beyond the area of application at concentrations of 100 to 8000 μg/mL suggesting that elemental sulphur may also act at a distance. This extra zone of inhibition increased in radius as the concentration of elemental sulphur increased (Fig. 5.16 a and c). The extended inhibition did not occur in the other species tested (Fig. 5.16 b and d). It was also noted that for all sulphur-sensitive fungi tested by this

bioassay, growth appeared denser around the perimeter of the inhibition zones giving a dark halo effect (Fig. 5.16). For most fungi this occurred most obviously around the application zones of the higher sulphur concentrations of 100 to 8000 µg/mL. However for *C. lindemuthianum*, *C. fulvum* and *V. dahliae* isolates where there was extended inhibition at high sulphur concentrations these dark halos appeared to be more prominent at lower concentrations of 12.5 to 50 µg/mL.

The patterns of inhibition once established did not change even after 40d in all TLC bioassays apart from a slight darkening of the halos surrounding zones of inhibition. This suggested that mycelial growth (from surrounding germinated spores) into sulphur treated zones was also inhibited at the concentrations of sulphur quoted above.

5.3.3 Inhibition of mycelial growth by elemental sulphur: Disc bioassay

The disc bioassay was designed to investigate the inhibition of fungal mycelial growth by elemental sulphur. All fungal pathogens were tested by this bioassay with the exception of *U. maydis* and *E. graminis*. For *Verticillium* and *Fusarium* spp. the disc bioassay was first attempted on Czapek dox agar. However if the fungus would not grow over the control discs on this medium PDA was used. *F. oxysporum* (all formae speciales) and *P. palmivora* did not grow over control discs on either Czapek dox or PDA and were therefore excluded from the disc bioassay. All other fungi tested grew on control discs to some extent, although not always as well as they grew on the agar between the discs (note the scores of 3 and 4 for some fungi even on control discs (Table 5.11).

All fungi showed a gradual reduction in the amount of disc colonised as the concentration of elemental sulphur increased (Table 5.11; Fig. 5.17). For all *V. dahliae*, *V. albo-atrum*, and *V. longisporum* isolates, *V. nubilum*, *F. avenaceum*, *V. lecanii*, *V. fungicola*, *V. chlamydosporium* and *S. nodorum* initial inhibition of mycelial growth was detected at 3.125 µg/mL. *V. tricorpus* appeared more resistant with initial inhibition occurring at 25 µg/mL and *M. fructigena* (Fig. 5.17) and *C. fulvum* even more resistant with initial inhibition occurring at 50 and 100 µg/mL respectively. *C. lindemuthianum* was the most susceptible fungus with initial inhibition at 0.78 µg/mL. In most cases growth on the discs had decreased to zero by 1000 µg/mL elemental sulphur. In the few cases where there was still a

small amount of growth on the discs at 1000 to 10 x 8000 μg/mL it was unclear as to whether the growth was actually on the discs or whether the fungus was growing up and over them. In some cases (*eg. V. dahliae* isolates) if the fungus was incubated for a long period of time on the agar, it appeared to start to grow onto the discs even at the highest sulphur concentrations, but on closer examination the fungus grew firstly around the perimeter of the discs and over rather than on the discs, essentially avoiding contact with the sulphur by bridging it.

Pathogen	Medium	Elemental sulphur (μg/mL)													
		Control	Solvent control	0.78	1.56	3.125	6.25	12.5	25	50	100	500	1000	8000	10 x 8000
V. dahliae (tomato isolate)	Czapek dox agar	5	5	-	5	4	4	3	3	2	2	1	1	1	1
V. dahliae (strawberry isolate)	Czapek dox agar	5	5	5	5	4	4	3	2	1	1	0	0	0	0
V. dahliae (cotton isolate)	Czapek dox agar	4	4	4	4	3	3	2	2	1	1	1	0	0	0
V. dahliae (cocoa isolate)	Czapek dox agar	3	3	3	3	2	2	2	1	1	0	0	0	0	0
V. albo-atrum (tomato isolate)	Czapek dox agar	3	3	-	3	2	2	2	1	1	0	0	0	0	0
V. albo-atrum (lucerne isolate)	PDA	3	3	3	3	2	2	2	2	1	1	1	1	0	0
V. longisporum (oil seed rape isolate)	Czapek dox agar	4	4	4	4	3	3	3	2	2	2	1	1	1	1
V. longisporum (horseradish isolate)	PDA	3	3	3	3	2	2	2	2	2	1	1	1	0	0
V. nubilum	PDA	5	5	5	5	4	4	3	2	2	1	1	0	0	0
V. tricorpus	PDA	4	4	4	4	4	4	4	3	3	3	2	2	1	1
V. theobromae	PDA	4	4	4	3	3	3	2	2	1	1	0	0	0	0
F. avenaceum	PDA	5	5	5	5	4	3	2	1	1	1	0	0	0	0
V. lecanii	Czapek dox agar	5	5	5	5	4	3	3	2	2	2	1	1	0	0
V. fungicola	PDA	4	4	4	4	3	3	2	2	1	0	0	0	0	0
V. chlamydosporium	Czapek dox agar	4	4	4	4	3	3	3	3	2	1	1	1	0	0
C. fulvum	PDA	4	4	-	4	4	4	4	4	4	2	2	1	0	0
M. fructigena	PDA	4	4	-	4	4	4	4	4	3	3	1	0	0	0
C. lindemuthianum	PDA	4	4	3	3	3	2	2	2	2	1	1	0	0	0
S. nodorum	CzV8CS agar	4	4	-	4	3	3	3	3	3	2	2	1	1	1

Table 5.11 Inhibition of fungal mycelial growth on discs impregnated with elemental sulphur. Antibiotic assay discs were impregnated with fifty μL of a sulphur solution and applied to agar plates. The plates were inoculated with a fungus and incubated until the leading edge of the fungus between the discs had grown beyond the outer edge of the discs. Each plate and corresponding sulphur concentration was examined visually to assess the pathogen's ability to grow over the treated discs and assigned a score of 0 to 5 with 0 representing no coverage and 5 for total colonisation.

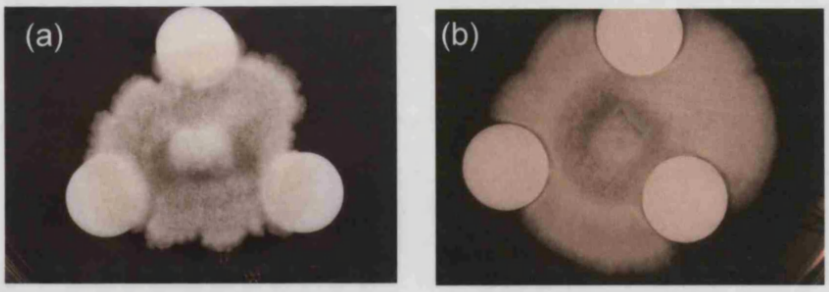

Figure 5.17 Disc bioassay showing toxicity of elemental sulphur to *M. fructigena* mycelial growth. Plate (a) shows antibiotic assay discs treated with pure dichloromethane and plate (b) shows discs treated with 50 μL of a 8000 μg/mL sulphur solution. Fungal growth can be seen on all control discs but not on sulphur treated discs.

5.3.4 Toxicity of elemental sulphur to bacteria and *U. maydis* in liquid culture

The shaken culture bioassay was designed to investigate the toxicity of elemental sulphur to fungal and bacterial multiplication. All bacterial pathogens and basidiospores of the fungus *U. maydis* were tested by this bioassay.

Multiplication of the Gram-negative bacteria *P. syringae* and *R. solanacearum*, and the Gram-positive bacterium *B. cereus* was not greatly affected by the presence of elemental sulphur. Bacteria in controls and in all concentrations of sulphur multiplied from 10^5 to $10^8/10^9$ bacteria/mL for *P. syringae* and *R. solanacearum* and from 10^4 to 10^7 bacteria/mL for *B. cereus* (Fig. 5.18). However a slight decrease in growth can be noted for all three bacteria by the middle concentrations of elemental sulphur (*ca.* 3.125 to 25 μg/mL).

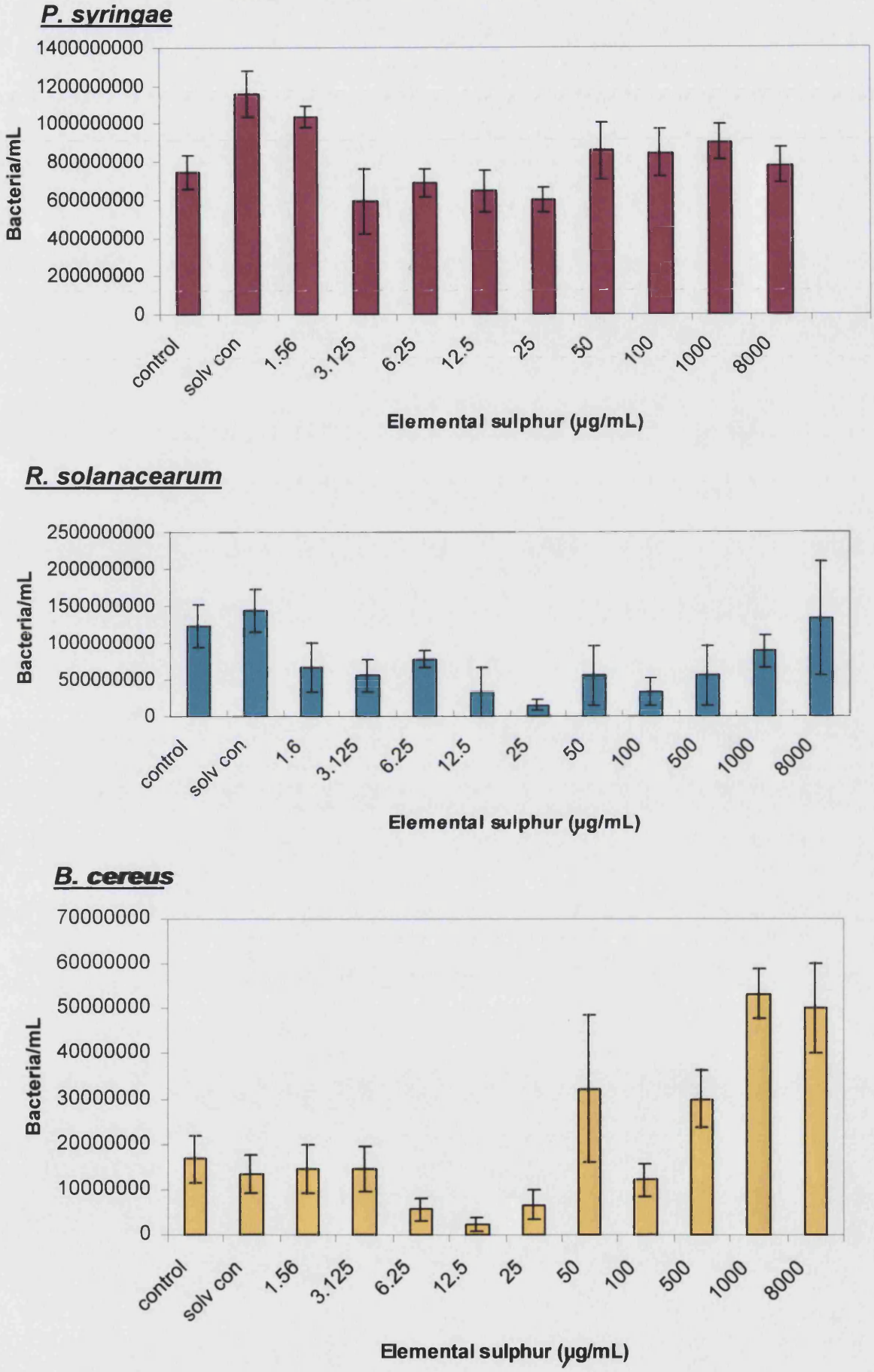

Figure 5.18 Toxicity of elemental sulphur to *P. syringae*, *R. solanacearum* and *B. cereus*. 1 mL of each sulphur solution was pipetted into three replicate vials, the solvent evaporated off and 1 mL of a bacterial suspension added (10^5 bacteria for *P. syringae* and *R. solanacearum* and 10^4 bacteria for *B. cereus*). Tubes were incubated over night and a viable count performed on each tube. Values represent mean number of bacterial cells present at each sulphur concentration with SE.

Basidiospores of *U. maydis* multiplied from 10^5 basidiospores/mL to 1.73×10^7 in the control and to 1.56×10^7 in the solvent control. In all concentrations of elemental sulphur there was no trace of basidiospores. They appeared to have been lysed in the presence of even the lowest concentrations of elemental sulphur.

5.4 Discussion

As for other phytoalexins, in order to implicate elemental sulphur production in the defence of plants against invading pathogens it must be present in the right place, in sufficient quantities and at the right time to inhibit the pathogen (Osbourn, 1999). In chapter 3 it was shown that elemental sulphur was produced by tomato in response to *V. dahliae* and *R. solanacearum*, by cotton in response to *V. dahliae*, by tobacco in response to *F. oxysporum* f. sp. *nicotianae* and by French bean in response to *F. oxysporum* f. sp. *phaseoli*. In all of these examples elemental sulphur was produced in the resistant interaction at the correct time to be involved in defence. In the tomato vs. *V. dahliae* incompatible interaction, sulphur was also shown by SEM-EDX to be localised in structures that would be of direct relevance to resistance against these vascular-invading pathogens. Many fungi can metabolise sub lethal levels of elemental sulphur (Beffa, 1993a) and so the presence and persistence of sulphur accumulations suggested fungitoxic levels. However, the levels of elemental sulphur detected in the plant tissue were not directly compared to those required to inhibit the pathogen. Here, using bioassays to establish toxicity to fungal spore germination (slide bioassay and TLC bioassay) and hyphal growth (TLC bioassay and disc bioassay), it is shown that elemental sulphur is toxic to all of the fungal pathogens involved in the interactions above. But, using a bioassay to determine cell division in liquid culture (shaken culture bioassay), even very high levels of elemental sulphur was not inhibitory to the multiplication of the vascular bacterial pathogen of tomato, *R. solanacearum*.

In the resistant interaction between tomato and *V. dahliae,* levels of elemental sulphur in total tissue extracts, which after 14 and 21 dpi were approximately 6 and 10 μg/g respectively, were greater than that required for inhibition of *V. dahliae* spore germination and hyphal growth as determined by the bioassays (assuming 1g of plant tissue = 1 mL sulphur solution). This agreed with data of Resende et al. (1996), who showed using a similar slide bioassay that purified elemental sulphur extracted from the cocoa plant was highly toxic to *V. dahliae* spores at concentrations well below those found *in vivo*. Furthermore, toxicity was found to occur at values similar to those described here (ED_{50} germination of 4.6 μg/mL and ED_{50} germ tube growth of 1.7 μg/mL found by Resende et al. in comparison to ED_{50} germination and germ tube growth both within the range 1.56 – 3.125 μg/mL found here). Although statistical analysis was not possible (the

reasons for which are explained in more detail later), there appeared to be negligible differences in the toxicity of elemental sulphur towards the *V. dahliae* blastospores and conidia, both of which may be produced during invasion of the plant vascular tissue (Schnathorst, 1981). Therefore, it seems that both conidia and mycelial growth of *V. dahliae* in the vascular tissue of tomato plants would be highly susceptible to the elemental sulphur produced. To conclude, it appears that elemental sulphur is produced in the right place, at the right time, and in sufficient quantities in resistant tomato plants to be implicated in the defence of tomato to *V. dahliae*.

Tobacco, French bean and cotton plants challenged with incompatible vascular fungi contained less elemental sulphur in total tissue extracts (100-250 ng/g maximum) than tomato challenged with *V. dahliae*. According to the bioassays this would not be enough to inhibit either spore germination or hyphal growth of the fungus to which they are resistant. However, it has been shown with other phytoalexins, for example phaseollin in bean, that whole tissue extracts give a gross underestimation of the actual amounts of phytoalexins present because phytoalexins become concentrated in hypersensitive cells, where they can be in considerable excess above that required for pathogen inhibition. (Bailey and Deverall, 1971). SEM-EDX analysis performed on resistant tomato xylem challenged with *V. dahliae* (chapter 3) revealed a general accumulation of sulphur all over the vascular tissue, although more intense localisations were also detected in distinct XP cells, gels, tyloses and vessel walls. This suggested that elemental sulphur might also be concentrated, like phaseollin.

Elemental sulphur was also detected in response to the vascular invading bacterium, *R. solanacearum,* albeit at lower levels than in response to *V. dahliae* (maximum of 200 ng/g in comparison to 10 μg/g); but elemental sulphur was not toxic to this pathogen even at very high concentrations (8 mg/mL). A similar situation was seen with phaseollin where several bacteria caused only small amounts of phaseollin to form in pods of French bean in comparison to that induced by fungi but phaseollin only had a slight effect on the growth of bacteria (Bailey and Deverall, 1971; Cruickshank and Perrin, 1971). It may be that elemental sulphur is produced in the xylem in response to both bacterial and fungal elicitors possibly as part of a general multi-component response to vascular challenge. It is common in plant defence for different components such as the

generation of ROS, changes in the plant cell wall, the induction of tyloses and the production of different phytoalexins, to act together in a co-ordinate fashion to contain infection. Of all of the defence mechanisms activated not all are necessarily detrimental to the invading pathogen, but in combination provide defence against a wide range of pathogens (Mansfield, 2000; Dixon, 2001). That is not to say that all phytoalexins are specific only to fungi. Although there have been fewer investigations of the toxicity of phytoalexins to bacteria, there are several phytoalexins that are antibacterial. For example cotton cells undergoing HR produce lacinilene C and 2,7 dihydroxycadalene at levels that are much greater than that required to inhibit bacterial growth of *Xanthomonas campestris* pv. *malvacearum in vitro* (Essenberg et al., 1992; Pierce et al., 1996) and the phytoalexin rishitin from potato is toxic towards *Erwinia atroseptica.* Furthermore, both bacteria and fungi are susceptible to the *Arabidopsis* phytoalexin, camalexin, although again the concentration required to inhibit fungi was 10 times less than that required to inhibit all but one of the bacteria tested (Rogers et al., 1996).

The literature on elemental sulphur toxicity to plant pathogens is confusing and incomplete. Therefore a survey was undertaken with the four bioassays described above to assess the effect of elemental sulphur on a wide range of fungi (from different classes) and bacteria (both Gram-negative and Gram-positive).

Initially, different species, isolates and formae speciales of *Verticillium* and *Fusarium* were investigated. Some of these were strong vascular pathogens such as *V. dahliae*, *V. albo-atrum, V. longisporum* and certain formae speciales of *F. oxysporum*, some were weak vascular pathogens such as *V. tricorpus* and *V. nubilum,* and some could not colonise the vascular tissues of host plants and instead caused rot diseases such as *V. theobromae, F. avenaceum* and three formae speciales of *F. oxysporum.* An investigation of elemental sulphur toxicity to these fungi would determine whether those fungi that could colonise the vascular systems of plants and therefore may come into contact with elemental sulphur (elemental sulphur is produced in the susceptible interactions but much later in infection when the fungus has already become established) were more resistant to this sulphur than those fungi that do not or cannot colonise the vascular systems of plants. Many plant pathogens have the ability to tolerate phytoalexins in their hosts either by degradative or non-degradative mechanisms as may be predicted if a pathogen were constantly exposed to a phytoalexin (VanEtten et al.,

2001). For example all alfalfa pathogens could detoxify the phytoalexin medicarpin using a variety of different enzymatic reactions (VanEtten et al., 1995). This plant resistance mechanism may then become a virulence trait for the pathogen (VanEtten et al., 2001). In several studies it has been shown that natural variants of pathogens, which either did not detoxify their host phytoalexin, or did so more slowly, or were more sensitive to it, were less virulent (VanEtten et al., 1995; Sbaghi et al., 1996; Suleman et al., 1996; Weltring et al., 1998; Morrissey and Osbourn, 1999). Thus, mutants of *Nectria haematococca* that were no longer able to detoxify the pea phytoalexin pisatin and the chickpea phytoalexins medicarpin and maackiain were much less virulent on hosts (Wasmann and VanEtten, 1996; Enkerli et al., 1998). Furthermore, the medicarpin/maackiain detoxification gene from *N. haematococca* has been transformed into *Colletotrichum destructivum* conferring on it pathogenicity to alfalfa (VanEtten et al., 2001).

To investigate whether elemental sulphur tolerance may be a virulence component during the infection of plants by vascular pathogens, the inhibition of fungal spore germination, germ tube growth and hyphal growth of xylem invading and non-xylem invading *Verticillium* and *Fusarium* spp., isolates and formae speciales by elemental sulphur in bioassays was compared. The data from spore germination and germ tube growth bioassays was subjected to probit analysis, the method commonly used to determine the potency of a toxin in a bioassay (Finney, 1964). However this data did not fit a typical dose response. Instead it appeared that germination remained high until a defined concentration of sulphur where almost complete inhibition of germination occurred. Elemental sulphur was applied to the slides in an organic solvent that was subsequently evaporated, causing the sulphur to form a layer of crystals on to which the fungal spore suspension was added. From subsequent analyses of fungal spore germination assessed microscopically, it seemed that at a certain density of crystals, elemental sulphur became highly toxic. This effect of density of sulphur particles has been previously noted regarding its toxicity to fungal spores and also for other fungicides such as copper (Wilcoxon and McCallan, 1931; Horsfall, 1956). Failure to conform to probit analysis also meant that data could not be analysed to give an accurate ED_{50} and instead it was said to fall between two values. Furthermore, data were not amenable to any other statistical analysis by which the different fungi could be compared in order to determine whether they differed in susceptibility to elemental

sulphur (K. Ward pers. comm.). However general trends were revealed. ED_{50} germination values for all *Verticillium* and *Fusarium* pathogens were between 1.56 and 3.125 μg/mL. For all *Fusarium* spp. whether vascular or non-vascular pathogens, and many *Verticillium* spp. including strong vascular and weak vascular pathogens, ED_{50} germ tube growth also fell within this range. Of the few exceptions there were both vascular and non-vascular *Verticillium* spp. whose germ tube growth were more susceptible and less susceptible to elemental sulphur and a weak vascular pathogen that was more susceptible. These data suggested that there was no link between the ability of the fungus to colonise the vascular system of the plant and elemental sulphur tolerance. Resende et al. (1996) showed that germ tube growth of *V. dahliae* of cocoa was more susceptible to pure extracts of elemental sulphur from the cocoa plant than germination rates. However both ED_{50} germination and ED_{50} germ tube growth stated by Resende et al. fell close to the range of ED_{50} values quoted here for both criteria, meaning that if there were a difference in susceptibility of spore germination and germ tube growth it would not necessarily be differentiated here. In the work by Resende et al. (1996) it is not stated whether the data fitted the probit scale and instead linear regression was used to calculate ED_{50}. Using linear regression may be inaccurate if the data follow the same pattern as that presented here. Anomalously, work by Barker et al. (1920) showed that elemental sulphur had no effect on spore germination of *Verticillium* spp.. In this study by Barker et al. hanging drops of water containing fungal spores were dusted with elemental sulphur until the outside of the film was completely covered and the spores left to germinate. However, the concentration of sulphur applied to the drop was not quantified and may be below that required for toxicity. Alternatively, perhaps the amount of contact that could occur between the spores and the elemental sulphur differed between the bioassay used by Barker et al. (1920) and that used here, accounting for the variation in results. For example the hydrophobicity of elemental sulphur may cause it to remain on the outside of the drop separate from the spores. This presents a very important point that a considerable amount of variation in the results may occur when determining the susceptibility of a pathogen to a phytoalexin due to differences in the bioassays used.

All TLC bioassays on *Verticillium* and *Fusarium* pathogens gave complete inhibition at the same elemental sulphur concentration (12.5 μg/mL) except for the

vascular pathogen *F. oxysporum* f. sp. *lycopersici* and the non-vascular pathogen *F. oxysporum* f. sp. *tulipae,* which were less susceptible. Finally, for all those fungi that could be tested by the disc bioassay (no formae speciales of *F. oxysporum* would grow over control discs), all showed an initial effect at the same concentration of elemental sulphur (3.125 μg/mL); exceptions were the weak vascular pathogen *V. tricorpus* that was less susceptible and the non-vascular pathogen *V. theobromae* that was more susceptible. Therefore it appears that *Verticillium* and *Fusarium* pathogens are very similar in susceptibility to elemental sulphur. Although there were a few pathogens that showed subtle differences in elemental sulphur susceptibility for each bioassay no one pathogen was ever found as consistently more susceptible or more resistant in more than one of the bioassays.

It seems that those *Verticillium* and *Fusarium* spp. that colonise the vascular systems of plants are no more tolerant to elemental sulphur than those that do not colonise the vascular system. This is perhaps not surprising as many pathogens are susceptible to their host phytoalexins. In the first evaluation of the antimicrobial spectrum of a phytoalexin, that being pisatin, Cruickshank found that plant pathogens were no more tolerant of their host phytoalexin than were other microorganisms (Cruickshank, 1962). To the author's knowledge no phytopathogenic bacterium has as yet been found that is more tolerant of its host phytoalexin than are other bacteria, and although tolerance does occur in fungi many also appear to lack tolerance to their host phytoalexins. For example, the aggressive pea pathogen *Aphanomyces euteiches* is highly sensitive to pisatin (Pueppke and VanEtten, 1976). Strangely some pathogens such as *A. euteiches* appear to be able grow *in vivo* in concentrations of pisatin many times greater than that needed to completely inhibit their growth *in vitro* and how this occurs is uncertain (Pueppke and VanEtten, 1976; VanEtten et al., 2001). It may be that some pathogens are physically separated from the phytoalexin but this does not seem to apply for *A. euteiches* (Pueppke and VanEtten, 1976). In the case of *V. dahliae* and elemental sulphur, it appears from total tissue extracts of tomato that elemental sulphur does not reach high enough concentrations to inhibit the pathogen in the susceptible interaction. Furthermore, even if elemental sulphur were to reach toxic concentrations by accumulation in defined structures as suggested previously, sulphur may be produced too late when colonisation has already been established and the fungus has moved on, whereas in the resistant

interaction plants produced sulphur more rapidly and to greater amounts to prevent colonisation. It is difficult to see why most pathogens haven't developed tolerance mechanisms to host phytoalexins when compared to how readily microorganisms acquire tolerance to antibiotics and fungicides. It has been suggested that it is perhaps disadvantageous to be too aggressive a pathogen, as eliminating the host would eliminate the habitat of the pathogen. Perhaps pathogens that acquire phytoalexin tolerance are less likely to survive because they destroy their host whereas those that lack tolerance mechanisms would be maintained (VanEtten et al., 2001). In addition, despite the use of elemental sulphur as a fungicide for so long, to the author's knowledge there has been no reported cases of field resistance suggesting that tolerance to it may be difficult or impossible to acquire or lethal to the pathogen.

To complete the survey a range of other fungal (Imperfect, Ascomycete, Oomycete, Basidiomycete) and bacterial (Gram-positive and Gram-negative) plant pathogens were investigated for susceptibility to elemental sulphur. Powdery mildew of barley, *E. graminis* also known as *Blumeria graminis* was chosen to represent this group, control of which is the earliest and best known application of elemental sulphur (Sharvelle, 1961; McGrath and Johnson, 1986; Jolivet, 1993). The obligately biotrophic nature of *E. graminis* prevented deployment of TLC and disc bioassays, but *E. graminis* conidia would germinate on clean glass slides in the presence of high humidity (Carver and Ingerson, 1987). High sensitivity to elemental sulphur was confirmed as spore germination was inhibited almost completely in the presence of the lowest concentration of sulphur (0.78 μg/mL). Although it is apparent that *E. graminis* was more susceptible than *Verticillium* and *Fusarium* spp. in this slide bioassay, it must be remembered that the *E. graminis* spores were applied dry, directly onto the elemental sulphur crystals. The *E. graminis* conidia may therefore be in closer contact with the sulphur. Again such differences in bioassays must always be taken into account as previously noted for the variation in results between bioassays for *Verticillium* spp.. Elemental sulphur was found to be toxic to uredospores of *Puccinia antirrhini* when applied dry but not toxic when applied in water (Doran, 1917).

C. fulvum (Imperfect), *S. nodorum* (Imperfect), *C. lindemuthianum* (Imperfect), *M. fructigena* (Ascomycete) and *P. palmivora* (Oomycete) were also tested for susceptibility to elemental sulphur by the slide, TLC and disc bioassays. All but *P.*

palmivora were highly susceptible to elemental sulphur. In general it appeared that *C. fulvum* and *C. lindemuthianum* were the most sensitive fungi, agreeing with work by Barker et al., (1920) who found that spores of *C. fulvum* were more susceptible to elemental sulphur than *Verticillium* spp.. *S. nodorum*, *Verticillium* spp. and *Fusarium* spp. all appeared very similar in susceptibility to elemental sulphur and *M. fructigena* which is generally known to be susceptible to elemental sulphur (Marsh, 1929) appeared to be slightly more resistant than the others to the sulphur. Such differences in the sensitivity of fungi to different phytoalexins have been apparent since the earliest investigations (Cruickshank and Perrin, 1961). However *P. palmivora* was completely unaffected by elemental sulphur with germination of zoospores and germ tube growth equivalent to controls at all concentrations of sulphur. Growth also occurred over all concentrations of elemental sulphur on TLC plates. Growth over discs impregnated with sulphur could not be assessed, as *P. palmivora* would not colonise control discs. To the author's knowledge, toxicity of elemental sulphur to this pathogen or any other Oomycete has never before been investigated. The Oomycetes are classed as fungal-like organisms or pseudofungi. They are not thought to be close relatives of the true fungi as shown by details of their flagella structure and a comparison of their small subunit rRNA and they appear to be more closely related to algae. However they can seem indistinguishable from true fungi in the form of their thalli and their ecological roles. These features along with profound economic effects of plant-pathogenic species mean that they are still studied by mycologists (Berbee and Taylor, 1999). It would be interesting to see if all Oomycetes were resistant to elemental sulphur. Oomycetes are certainly not resistant to all phytoalexins as glyceollin produced by soybean is highly toxic to *Phytophthora megasperma* var. *sojae* (Yoshikawa et al., 1978), the pepper phytoalexin capsidiol is toxic to *Phytophthora capsici* (Garcia-Perez et al., 1998) and the potato phytoalexin rishitin is toxic to *Phytophthora infestans* (Harris and Dennis, 1977).

U. maydis grows in a yeast-like form in culture by budding to produce basidiospores and so the shaken culture bioassay was used to assess fungal growth in the presence of elemental sulphur. This fungus was shown to be highly susceptible to elemental sulphur as all spores lysed in the presence of even the lowest concentration of sulphur. No previous record could be found on the toxicity of elemental sulphur to maize smut, but previous field trials to determine whether elemental sulphur could be used to control sorghum grain smut, *Sphacelotheca*

sorghi and closed smut disease of barley (*Ustilago hordei*) revealed it as highly effective in controlling the disease (Uppal and Malelu, 1928; Jones, 1934). It has also been previously recommended for onion smut control (Thatcher and Streeter, 1925) and so the fungicidal activity of elemental sulphur to smuts is already well known. Again it is tempting to suggest that *U. maydis* is more susceptible than most fungi to elemental sulphur, but again the bioassay used was very different to the bioassays used on the other fungal species and may be the cause of this variation.

Bacterial susceptibility to elemental sulphur analysed by this same shaken culture bioassay revealed that like *R. solanacearum,* neither growth of the Gram-negative bacterium *P. syringae*, nor that of the human Gram-positive bacterium *B. cereus*, were highly susceptible to elemental sulphur. All were able to grow two to three orders of magnitude in the presence of all concentrations of sulphur. It is possible that elemental sulphur has a slight effect on growth rate at concentrations of 3.125 to 25 μg/mL as bacterial multiplication did not reach the same level as controls, but this effect was considered minimal as substantial growth still occurred. Gram-positive bacteria are generally more sensitive to phytoalexins and antibiotics (Smith, 1982; Smith and Banks, 1986), however in this case both appeared similarly insensitive. The same was found to be true with the *Arabidopsis* phytoalexin camalexin, where the Gram-positive bacterium *Listeria monocytogenes* was no more susceptible than the Gram-negative bacteria *P. syringae* pv. *maculicola* and *Escherichia coli*. As mentioned above, there are several phytoalexins that are toxic to bacteria but there seems to be little previous literature of elemental sulphur toxicity to bacteria. The idea that bacteria are not susceptible to elemental sulphur is perhaps not surprising as many specialised bacteria can produce elemental sulphur as described in chapter 4. These include the purple and green sulphur bacteria such as *Chromatium vinosum* and *Chlorobium* spp.; these can accumulate globules of elemental sulphur either internally or externally as an intermediate during the oxidation of sulphide or thiosulphate to sulphate where reduced sulphur compounds are used as electron donors for anoxygenic photosynthesis. Also sulphur-oxidizing bacteria such as *Thiobacillius ferroxidans* can produce elemental sulphur aerobically where it accumulates in the outer membrane of the cell wall and in vacuole-like structures (Hazeu et al., 1988; Prange et al., 1999). However data by Izac et al. (1982) suggested that elemental sulphur was toxic to both the Gram-positive *Bacillus*

subtilis and the Gram-negative *Salmonella typhimurium*. Once again the bioassays used were different to the bioassay used here. Izac et al. (1982) used agar impregnated with elemental sulphur to a concentration of 2 mg/mL and streaked the test organism onto it. This completely inhibited the growth of both pathogens. It may be that these bacteria are more sensitive than the ones tested in this survey, or alternatively the variation may be due again to the different conditions of the bioassay.

There are a few anomalies of the bioassays that must also be explained. It can be noted that although all elemental sulphur-sensitive fungi showed reduction in germination and germ tube growth at concentrations up to 100 μg/mL in the slide bioassay, many showed increased germination and germ tube growth as the concentration of elemental sulphur was increased to 8000 μg/mL. This again seemed to be associated with the way in which the sulphur crystallised out of solution when the solvent was evaporated from slides. As mentioned previously, the density of crystals appeared to have a large impact on the toxicity of the sulphur causing a rapid decrease from high germination to almost complete inhibition of germination at a defined concentration. It was also evident that the size of the crystals was important in determining toxicity of elemental sulphur to the fungal spores. Thus, at concentrations ≤100 μg/mL where the sulphur particles produced were small and densely packed, germination was inhibited, but at higher concentrations where larger sulphur crystals were formed that were less densely distributed, fungal spore germination was partially restored and spores could be seen germinating directly on the sulphur crystals. The phenomenon of smaller sulphur particles being more toxic than larger particles to fungi, and the link between density of sulphur particles and toxicity have been noted many times in the literature (Barker and Wallace, 1921; Young, 1922; Thatcher and Streeter, 1925; Wilcoxon and McCallan, 1930; Wilcoxon and McCallan, 1931; Martin and Salmon, 1932; Horsfall, 1956). Furthermore, in the context of application to plants, small particle size is desirable because they adhere better than larger particles to leaves and are more effective *in vivo* (Wilcoxon and McCallan, 1931). Even in the bacterial growth experiments, which showed a slight decrease in growth rate in response to concentrations of elemental sulphur between approximately 3.125 to 25 μg/mL, growth was restored to equivalent levels to controls in the presence of high levels of elemental sulphur. In the case of *B. cereus*, high levels of elemental sulphur (>100 μg/mL) appeared to promote growth. Although this has yet to be

confirmed with replicate experiments, it may represent the ability of *B. cereus* to metabolise elemental sulphur. This could be tested by analysing the supernatant for a reduction in the concentration of elemental sulphur or by tracking metabolites of labelled sulphur.

In the TLC bioassays it was noted that growth appeared denser around the perimeter of the inhibition zones for all elemental sulphur-sensitive fungi giving a dark halo effect. It is thought that this may be due to lower, sublethal concentrations of elemental sulphur present in this area that could be metabolised by the fungus and used for growth (Beffa, 1993a). For most fungi this occurred most obviously around the application zones of the higher sulphur concentrations of 100 to 8000 μg/mL. However for *C. lindemuthianum*, *C. fulvum* and *V. dahliae* isolates these dark halos appeared to be more prominent at lower concentrations of 12.5 to 50 μg/mL. At concentrations above this, elemental sulphur appeared to have an effect at a distance from the application site with a band of thinner mycelial growth around the spot of complete inhibition. It has been noted previously that elemental sulphur could affect the development of fungal spores even at distance and this was suggested to be due to a toxic sulphur vapour (Doran, 1922). It is likely that this vapour was due to the release of very small particles of elemental sulphur from a deposited mass (Barker and Wallace, 1921). In the past horticulturalists have produced sulphur vapour by coating the hot water pipes of the glasshouses with sulphur paste forming a cloud of sulphur particles. This approach was later replaced by a vaporiser, that boiled sulphur under such conditions that the vapour, which evolved, condensed into a cloud of very finely divided elemental sulphur particles (Barker and Wallace, 1921). It is possible that similar small particles of elemental sulphur contaminated the areas surrounding application zones during the process of application of the sulphur to the TLC or alternatively from the vaporisation of elemental sulphur from the zones of application after they had been deposited. It seems that the fungal spores already implicated as most sensitive to elemental sulphur by the slide and TLC bioassays (*C. fulvum* and *C. lindemuthianum*) were also most sensitive to this effect. However *V. dahliae* isolates also showed this effect suggesting that *Verticillium* and *Fusarium* spp. may not be as similar in their response to elemental sulphur as originally thought. Also in the TLC bioassays, small "micro colonies" of fungal growth often appeared even at very high sulphur concentrations within zones of otherwise complete inhibition of fungal growth. This may also be related to the

crystallisation of elemental sulphur that perhaps leaves small areas free of sulphur between crystals for fungal growth to occur. Alternatively, it is possible that a small number of spores within a population can tolerate elemental sulphur. A small proportion of *C. lindemuthianum* sporelings placed onto agar containing inhibitory concentrations of phaseollin developed into colonies. Within these sporelings just a few cells survived initial exposure to phaseollin and grew, and it was suggested that these cells may either differ in their initial sensitivity to phaseollin or may have adapted to grow in its presence (Skipp and Bailey, 1976).

In conclusion, elemental sulphur was toxic to all but one of the fungi tested in this survey, i.e. *C. fulvum, C. lindemuthianum, M. fructigena, S. nodorum, E. graminis, U. maydis* and *Verticillium* and *Fusarium* spp.. Interestingly *P. palmivora* was highly tolerant of elemental sulphur as were all of the bacteria. In the interactions between *V. dahliae* and resistant cocoa and tomato plants it has now been demonstrated that the levels of elemental sulphur produced *in vivo* are theoretically sufficient to inhibit the invading pathogen (Resende et al., 1996). In both interactions this has enhanced the evidence for the role of elemental sulphur in plant defence. Furthermore, it can be noted that toxicity of elemental sulphur to all susceptible fungi fell within the order of magnitude noted by Smith (1982) as generally common for organic phytoalexins in *in vitro* assays. All *Verticillium* and *Fusarium* spp. and *S. nodorum* seemed very similar in their susceptibility to elemental sulphur despite their different modes of infection that may or may not result in exposure to elemental sulphur. The other fungi varied slightly in susceptibility to elemental sulphur with spores of *C. fulvum, C. lindemuthianum, E. graminis* and *U. maydis* appearing particularly vulnerable and spores of *M. fructigena* appearing more tolerant; however, *U. maydis* and *E. graminis* cannot strictly be compared to the other fungi in view of differences in the bioassays used. None of the fungi could be compared statistically for differences in susceptibility to elemental sulphur because the data did not fit the criteria for probit analysis.

Differences between Oomycetes, bacteria and true fungi may provide clues as to why *P. palmivora* and bacteria are resistant to elemental sulphur and other fungi are susceptible. Tolerance to phytoalexins can be classed in two main groups, as briefly mentioned earlier. Firstly enzymatic or non-enzymatic detoxification may occur such as the detoxification of the pea phytoalexin pisatin by pisatin

demethylase of *N. haematococca* (VanEtten et al., 2001). It is possible that elemental sulphur may be enzymatically or non-enzymatically degraded by *P. palmivora* and bacteria. Bacteria, fungi and higher plants can metabolise elemental sulphur although the mechanisms by which they do so are not well understood in any of these cases. Both enzymatic and non-enzymatic routes have been suggested (Jolivet et al., 1992; Beffa, 1993a; Pattaragulwanit et al., 1998). Secondly, non-degradative tolerance mechanisms may account for the tolerance of a pathogen to a phytoalexin, such as an inability to take up the phytoalexin, or a mechanism to remove the phytoalexin from the cell before it causes damage. For example *N. haematococca* as well as possessing a degradative mechanism to break down pisatin, is also thought to possess non-degradative tolerance to pisatin that involves a change in pisatin retention by the cells (Denny et al., 1987). Furthermore, fungicide-resistant *Aspergillus nidulans* that also has enhanced resistance to pisatin showed up-regulation of an ATP-binding cassette transporter that may be involved in pumping the phytoalexin out of the cell (Del Sorbo et al., 1997). Many non-degradative tolerance mechanisms involve the cell membrane (VanEtten et al., 2001) and in some cases sterols have been implicated; for example ergosterol mutants of *Neurospora crassa* were hypersensitive to pisatin (Kasbekar and Papavinasasundarum, 1992; Papavinasasundarum and Kasbekar, 1993). In most fungi ergosterol is the main sterol in the cell membrane whereas in the Oomycetes, like mammals, cholesterol is the dominant sterol (Robson, 1999). The majority of prokaryotes have no sterols in their membranes (Madigan et al., 1997). Coincidentally, Oomycetes and bacteria are highly resistant to saponins *in vitro* because of the differences in levels of membrane sterols (Arneson and Durbin, 1968) and so perhaps differences such as these may be involved in non-degradative tolerance to elemental sulphur.

With respect to determining why certain organisms are more susceptible than others to elemental sulphur it would also be useful to know how elemental sulphur exerts its toxic effect on fungi. Conversely, these differential effects may hold the key to explaining it elusive mode of action. The mechanism of toxicity has been an issue of much debate over the last century and has included some very imaginative and speculative hypotheses (Tweedy, 1981). For example, Mangini (1871) suggested that electricity was generated as a result of the contact of sulphur with the plant and this inhibited growth of the fungus, and Mach (1879) suggested that sulphur collected and concentrated the rays of the sun, which in

turn generated sufficient heat to burn the fungus. The mode of action of elemental sulphur is still uncertain but the leading current hypothesis is that fungal cells are permeable to S^0 and so it is taken up into the cytoplasm where it then has an effect on the mitochondrial respiratory chain. There may be transfer of hydrogen ions to S^0 instead of O_2 causing the production of toxic hydrogen sulphide as suggested by McCallen and Wilcoxon (1931) and Miller et al. (1953). Tweedy and Turner (1966) suggested interference of the mitochonidrial electron transfer at the level of cytochrome b and c. Furthermore Beffa et al. (1987; 1993b) showed that exogenous elemental sulphur was able to reduce itself at the level of cytochrome c and that reduced glutathione and other cellular sulphydryl groups could participate with minor importance to this reduction. Beffa et al. (1993b) also found that elemental sulphur could quickly and non-enzymatically oxidise proteic and non-proteic sulphydryl groups implicated in many respiratory functions of the mitochondria. These phenomena could produce a modification of the oxidation state of the respiratory complexes, disturb the electron flux in the mitochondrial respiratory chain and consequently alter oxidative phosphorylation causing fungitoxicity (Jolivet, 1993).

Finally, this chapter highlights some of the important problems associated with bioassays that are summarised below. Numerous bioassays have been used to assess the fungitoxic and bacteriotoxic activities of phytoalexins (Sharvelle, 1961; Hislop and Clifford, 1975; Bailey and Skip, 1978; Russell et al., 1992; Brantner et al., 1994; Cole, 1994). When the purpose of the bioassay is to help clarify the role of a phytoalexin in disease resistance as it was here, bioassays should be designed to yield accurate measurements of the effective concentrations of the phytoalexins and this should be related to the amount found in plant tissues. However, as previously explained, if the phytoalexin is concentrated within specific cells or structures of the plant tissue rather than evenly distributed it is difficult to determine the concentration of the phytoalexin to which the pathogen may be exposed as whole tissue extracts give a gross underestimation.

As highlighted throughout this discussion, variations in the bioassays used to determine toxicity of a phytoalexin to a target organism, can greatly influence the results obtained. Similar problems were noted when assessing the toxicity of phaseollin to *C. lindemuthianum*. In some tests inhibition was caused by less than 5 μg/mL but in others very little inhibition occurred at over 100 μg/mL (Bailey and

Deverall, 1971; Cruickshank and Perrin, 1971; Bailey, 1974). The methods used to assess toxicity can be infinitely variable. Various types of fungal growth can be used such as conidia, sporelings or hyphae; in liquid or solid media; in the presence or absence of added nutrients. Furthermore, different time scales can be applied to each bioassay that can last for min, h, or quite often several d. All of these factors may affect the result of the bioassay (Bailey and Skipp, 1978). It is tempting to compare directly the four bioassays used here in order to relate elemental sulphur toxicity to fungal mycelial growth, fungal spore germination and bacterial growth. For example with *C. fulvum* mycelia appear more tolerant to elemental sulphur than spores by interpreting results from the disc and slide bioassays. Comparison of data with that of other groups must also remain tentative as differences between bioassays may generate differences in apparent susceptibility. Despite application of the same amount of the same sulphur solutions to the slides and the TLC plates for the respective bioassays, the area that the sulphur covers differs between the two (diameter 10 mm and *ca.* 25 mm respectively) and so the concentrations cannot be compared. Elemental sulphur may also interact differently with the glass slide, the glass vials, the silica TLC plate and the antibiotic assay discs in the different bioassays. Furthermore, spores were applied to the slide bioassay in water but to the TLC and disc bioassays in nutrient containing media and the nutrient status can affect the ability of the fungus to overcome the phytoalexin. A few spores of *C. lindemuthianum* survived increased levels of phaseollin especially with increased nutrient status (Skipp and Bailey, 1976). In addition, the pH of the medium in which the fungus is exposed may also have an effect as alkaline conditions reduced the response of fungi to pisatin and kievitone (Deverall and Rogers, 1972; Bull, 1981). The timing at which growth measurements are taken can also be important. On agar impregnated with phaseollin, growth of *C. lindemuthianum* occurred in two phases; it was completely inhibited for an initial period and then grew at a similar rate to controls (Bailey et al., 1976). As a consequence of this, if a single assessment of growth were made then the colony diameter and therefore the noted effect of the phytoalexin would depend on when the assessment was made. For the slide, shaken culture and disc bioassays only one measurement of growth was made and this was at a different time point after exposure for each bioassay. This may cause variation in results if there is an effect like that of *C. lindemuthianum*. However for the TLC bioassay growth was assessed continually and the pattern of inhibition did not change substantially for more than 40d. Another consideration is

that different bioassays may allow a different amount of contact between the phytoalexin and the target organism that also may cause toxicity results to vary. For example, liquid media may allow accumulation of phytoalexins by hyphae whilst agar media may restrict their availability. It was found that phaseollin could kill spores within 2 min when incorporated into liquid media, whilst several h were needed for toxicity on agar media (Skip and Bailey, 1976). This would suggest that the disc bioassay was less sensitive than the slide bioassay.

It is important to note that many phytoalexins and fungicides like elemental sulphur are hydrophobic and so organic solvents are used as carriers. Here elemental sulphur was deposited onto the test substrate (slides, TLC, discs, vials) in dichloromethane, the solvent evaporated and the spores or bacteria added in water or media. As elemental sulphur does not dissolve in water, a true concentration cannot be assigned to the suspension and instead a pseudo-concentration is given. This is really a defined wt of sulphur crystals suspended in water. Such crystallisation causes problems in bioassays particularly if, like here, different sizes of crystal are differentially toxic resulting in a dose response curve with two peaks. Furthermore, these pseudo-concentrations may account for the unsuitability of the elemental sulphur spore germination toxicity data for probit analysis. Similar problems must occur with other hydrophobic phytoalexins such as wyerone and many of the other hydrophobic fungicides. These problems also raise the question of how or in what form a hydrophobic molecule such as elemental sulphur exists in a hydrophilic cellular environment *in vivo* and this will be discussed in more detail in chapter 6.

Clearly, *in vitro* bioassays provide only limited information regarding the toxicity of a phytoalexin *in vivo*. No *in vitro* bioassay can be presumed to reflect exactly how a phytoalexin acts *in vivo.* It is therefore useful to utilise a variety of bioassays to assess toxicity as carried out here. In view of the susceptibility of so many fungi to elemental sulphur both here and in the literature, and its use for so many years as a fungicide, the idea that elemental sulphur is toxic to numerous fungi is not in question. It is the amount required for toxicity in relation to the form that the sulphur takes in the plant, the amount present in the plant, the physiological environment in which the elemental sulphur occurs in the plant and the degree of contact that can occur between the sulphur and the fungus within the plant, that are the key issues requiring further investigation.

Chapter 6
General Discussion

The concept of phytoalexins as induced antimicrobial compounds has been continually developing since the work by Müller and Börger in the 1940s. It has taken over 60 years to gain even a basic understanding about this one component of the defence system of a plant, and the concepts surrounding phytoalexins are continually being altered as new information becomes evident. The production of elemental sulphur by plants and its possible involvement in plant defence as the first inorganic phytoalexin is a new phenomenon in the history of phytoalexin research. Following the initial discovery of elemental sulphur accumulation in a resistant line of the cocoa plant (Sterculiaceae) in response to *Verticillium dahliae* (Cooper et al., 1996; Resende et al., 1996), it has been shown here that the ability of plants to produce elemental sulphur is more widespread. S^0 accumulated in the vascular tissues of plants from several families (Solanaceae, Malvaceae, Leguminosae) in response to both bacterial and fungal vascular pathogens, and the pattern of accumulation resembled that of various organic phytoalexins, with a more rapid and intensive production in the resistant varieties than in the susceptible (Bailey and Deverall, 1971; McCance and Drysdale, 1975; Gentile and Matta, 1976; Hargreaves et al., 1977; Yoshikawa et al., 1978; Yoshikawa and Masago, 1982). However, elemental sulphur was not detected in the vascular tissues of strawberry (Rosaceae) or maize leaves (Gramineae) in response to a vascular fungus and bacterium respectively, or in the leaves of plants from various families (Solanaceae, Leguminosae, Brassicaceae, Compositae) in response to incompatible bacterial pathogens. It is therefore feasible that elemental sulphur production may be xylem specific as previously discussed and not produced by all plant species (chapter 3). Representative species from the plant families most relevant to agriculture have been assessed in this survey and so this part of the research is unlikely to be continued much further. However, it may be of interest to see whether other members of the Rosaceae and Gramineae families also lack the ability to produce elemental sulphur. Furthermore, the hypothesis that elemental sulphur production is xylem-specific needs confirmation. Leaves were challenged only with incompatible bacterial pathogens in this survey but an incompatible fungal pathogen was a better elicitor of elemental sulphur production in tomato vascular tissue. Therefore the interaction between tomato and

Cladosporium fulvum could be a suitable interaction to determine whether elemental sulphur could be produced in leaf tissue by fungal elicitors.

Arabidopsis leaves and *Brassica oleracea* cotyledons (Brassicaceae) contained constitutive levels of elemental sulphur. Whether this sulphur originated from the same biosynthetic pathway as that produced in response to pathogens, or whether it was produced by a different pathway, such as the breakdown of glucosinolates remains uncertain. A mutant screen to identify *Arabidopsis* plants unable to produce elemental sulphur would be the ideal way in which to investigate its biosynthesis and role in defence in this plant, but would require a rapid, facile detection system. The extraction and GC-MS procedure for detection of elemental sulphur would be too slow for such a screen. Some of the other methods of elemental sulphur detection mentioned previously (chapter 1) may be more suitable for this type of screen such as colorimetry (Bartlett and Skoog, 1954; Sörbo, 1957; Hazeu et al., 1988; Chan and Suzuki, 1993; Stefess et al., 1996; Henshaw et al., 1997) or TLC (Pezet and Pont, 1977; Krauss et al., 1984; Joyard et al., 1988; McLaughlin and Sherma, 1994; Ramsden, 1995).

Analogous to the cocoa vs. *V. dahliae* interaction, in the tomato vs. *V. dahliae* interaction sulphur accumulated in structures of direct relevance to resistance against xylem invading fungi as shown by SEM-EDX. However SEM-EDX cannot differentiate between bound sulphur and elemental sulphur. Therefore it is not certain whether the sulphur localised by SEM-EDX is elemental or in the form of another compound such as glutathione. In addition, *Brassica* spp. are sulphur-rich plants and coincidentally SEM-EDX showed sulphur to be present in all cell types within the leaves of *Arabidopsis*. Determination of the form of sulphur located by SEM-EDX could be attempted with techniques such as Ablation-MS or Time of Flight - Secondary Ion MS (ToF-SIMS). Both of these methods allow the collection of data from the surfaces of solid samples under ultra-high vacuum conditions by ablating the surface of the sample and analysing the secondary ions ejected by MS. Such techniques are being developed at UMIST (Manchester, UK) to analyse microbial cells for spatial localisation of microbial metabolites at the sub-μm scale and at the University of Bristol (Bristol, UK) for plant seed analysis (Cannon et al., 1997; Colliver et al., 1997; Pacholski et al., 1999; M. Beale pers. comm., IACR, Long Ashton, UK). Adaptations of such technology may also be used to detect

rapidly the presence or absence of elemental sulphur in *Arabidopsis* leaves if a mutant screen were to be performed (M. Beale pers. comm.).

If elemental sulphur were present in all areas of *Arabidopsis* leaves, it raises the question of how fungal pathogens, particularly necrotrophic fungi, are able to invade this plant, as all pathogens tested in the toxicity survey with the exception of the Oomycete *Phytophthora palmivora* were highly susceptible to elemental sulphur (chapter 5). Various fungal pathogens do invade *Arabidopsis* and although many are biotrophic such as *Peronospora parasitica*, *Plasmodiophora brassicae* and *Albugo candida* (Tang et al., 1996; Butt et al., 1998; Manzanares-Dauleux et al., 2001), or vascular such as *Verticillium longisporum* and *V. dahliae* (Steventon et al., 2001), there are also a few necrotrophic pathogens of *Arabidopsis* that include *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Govrin and Levine, 2000; Muckenschnabel et al., 2002). It may be that elemental sulphur is localised to certain structures within the plant cells and the pathogen does not come into contact with it during invasion. This may account for the ability of biotrophs but not of necrotrophs to invade *Arabidopsis*. It seems that only very aggressive strains of these necrotrophs are able to invade *Arabidopsis* (Govrin and Levine, 2000) and perhaps these strains are more tolerant to elemental sulphur than other fungi. None of these *Arabidopsis*-invading pathogens were tested in the toxicity survey, but it is likely that because they are Oomycetes, *P. parasitica* and *A. candida* are tolerant to elemental sulphur. It was discovered here that those *Verticillium* and *Fusarium* spp., isolates and formae speciales able to invade the vascular systems of plants (and hence possibly exposed to elemental sulphur during invasion) were no more tolerant to elemental sulphur than non-vascular isolates. This suggests that exposure to elemental sulphur does not necessarily lead to increased tolerance. However previously in toxicity bioassays *B. cinerea* has been shown to be more tolerant to elemental sulphur than several other fungi (Barker et al., 1920; Barker, 1929; Marsh, 1929; McCallan and Wilcoxon, 1931). Perhaps the toxicity survey should be extended to analyse these necrotrophic pathogens of *Arabidopsis* to determine if they show some degree of tolerance to elemental sulphur.

A further consideration regarding the presence of elemental sulphur in plant cells is how such a hydrophobic element exists within a mainly hydrophilic cellular environment *in vivo*. Even in the long-studied elemental sulphur-producing

bacteria this anomaly remains uncertain. Elemental sulphur is solid and usually crystalline at 20°C whereas sulphur globules formed by bacteria are liquid and amorphous (Steudel et al., 1990). When crystalline elemental sulphur is formed from aqueous sulphide in industrial chemical desulphurisation processes, first $HS^{\bullet}$ radicals are formed which exist at pH values near 7 mainly as $S^{\bullet-}$. Their spontaneous decay results in the formation of the disulphide ion S_2^{2-}. The further oxidation of this disulphide by either $S^{\bullet-}$ radicals or oxidation with transition-metal ions like Fe^{III} and Cu^{II} yields higher polysulphide ions from which the S_8 molecules are formed. In water these hydrophobic molecules form clusters, which grow to form droplets of liquid sulphur (sulphur sol). Depending on the composition of the aqueous phase, crystallisation of this liquid sulphur is rapid or delayed with surfactants causing delay of this crystallisation (Steudel, 1996). The elemental sulphur produced by sulphide-reducing bacteria is considered to be formed by a mechanism similar to that described above, although the liquid elemental sulphur produced is thought not to crystallise, hence remaining as globules (Steudel, 1996). Proteins may act as surfactants wrapping around the sulphur with their hydrophobic parts to the surface of the S_8 droplets and their hydrophilic moieties directed towards the aqueous environment thereby preventing crystallisation (Brune, 1995; Steudel, 1996). These proteins may attract a layer of water causing a change in the density of the sulphur globules and allowing the droplets to exist in a hydrophilic environment (Steudel, 1989; Steudel et al., 1990; Steudel, 1996).

The levels of elemental sulphur present in tissue extracts from both cocoa and tomato were shown to be toxic to *V. dahliae in vitro* (Resende et al., 1996; chapter 5). Therefore it appears that for both of these interactions elemental sulphur is produced in the right place, at the right time and in sufficient quantities in resistant varieties to fulfil the criteria by Osbourn (1999) and implicate elemental sulphur in defence against *V. dahliae*. But, the origin and biosynthetic pathway of elemental sulphur production in eukaryotes is by an uncharacterised pathway. The interaction between resistant tomato plants and *V. dahliae* was therefore used as the model to begin to investigate this phenomenon. Changes in the expression of genes of normal sulphur metabolism in plants were investigated and showed higher expression of a sulphate transporter, APS reductase, glutathione synthetase and cysteine synthase genes in the pathogen-inoculated xylem samples in comparison to the controls at 7 dpi only. These increases in expression

could be related to the subsequent transient increases in sulphate, and thiol levels in these tissues shown by S. Hall (IACR Rothamsted, Harpenden, UK) (Williams et al., 2002; appendix 3) and to the rapid accumulation of elemental sulphur (chapter 2). Furthermore cDNA-AFLP analysis performed by J. Howarth (IACR Rothamsted, Harpenden, UK) revealed a clone (6-5) that was up-regulated specifically in response to *V. dahliae* at all time points following infection and in all parts of the plant. Although this more molecular investigation did not resolve the pathway of elemental sulphur production it did allow the formulation of a possible model (chapter 4). This model involved the breakdown of glutathione *via* cysteinyl glycine to cysteine by the enzymes γ-glutamyl transpeptidase and a dipeptidase respectively, followed by the breakdown of cysteine to sulphide by cysteine desulphhydrase. Sulphide may then be oxidised to elemental sulphur by one of several mechanisms that includes oxidation by cytochromes, the action of a sulphide-quinone oxidoreductase or the action of a sulphide oxidase. All of the enzymes in this model are known to exist in higher plants except sulphide-quinone oxidoreductase and sulphide oxidase that have so far only been found in prokaryotes. However, none of these enzymes have been cloned completely and only γ-glutamyl transpeptidase has been purified in higher plants (Lancaster and Shaw, 1994; Martin and Slovin, 2000).

The molecular data presented here has provided many avenues of future research. The infection of resistant tomato plants with *V. dahliae* does appear to affect sulphur metabolism and this may be required for elemental sulphur production. There are more genes of sulphur metabolism (chapter 4) for which expression analysis can be carried out on in this system although some first require cloning from tomato or a close relative. It may also be relevant to look at the expression of glutathione reductase (Noctor et al., 1998a), GSTs (Edwards et al., 2000) and phytochelatin synthase (Hall, 2002) that are also believed to play a role in plant defence (chapter 4). This would give a wider picture of how sulphur metabolism is altered during pathogen challenge. As well as looking at genes and enzymes involved in sulphur assimilation and the production of glutathione, it would also be relevant to look in more detail at the degradation of glutathione and the enzymes involved, particularly the activity of cysteine desulphhydrase for which there is an assay developed (Schmidt, 1987; Burandt et al., 2001). Furthermore, the cysteine desulphhydrase inhibitor used by Schneider and

Rennenberg (1992) could be used in tomato challenged with *V. dahliae* to determine the effect on elemental sulphur production.

A study of how the expression of genes involved in sulphur metabolism is affected in susceptible tomato plants inoculated with *V. dahliae* is required to determine the specificity of any up- or down-regulation of gene expression in plant defence. In the susceptible interaction elemental sulphur was produced, but only much later when the plant was intensively colonised by the pathogen (chapter 3). Furthermore, no increases in cysteine or glutathione were detected in *V. dahliae*-inoculated susceptible tomato plants by HPLC in comparison to controls (Williams et al., 2002; appendix A3.2 and A3.3).

In parallel to the experiments on sulphate and thiol analysis with tomato plants supplied with high (1 mM) sulphate and challenged with *V. dahliae,* analyses were also carried out by S. Hall on plants fed with medium (0.1 mM) or low (0.02 mM) sulphate. Plants supplied with low sulphate were extremely chlorotic and stunted and so were not analysed further. Plants grown with medium sulphate appeared healthy but peaks of sulphate, cysteine and glutathione did not occur in response to the pathogen (S. Hall, unpublished data). It is not known as yet how elemental sulphur production is affected by sulphate nutrition. Since the reduction of sulphur in fertilizers by the introduction of sulphur-free urea and triple superphosphate and the development of environmental controls on industrial and aerial pollution (Roberts and Fisher, 1985; Jolivet, 1993), sulphur deficiency has become the most widespread nutrient disorder; this shortfall means that farmers are now often required to add sulphur to crops (Mengel, 1993; Schnug and Haneklaus, 1998; P. Williams pers. comm. Wilton Estate, Salisbury, UK). As well as a decrease in yield and a decline in crop quality, it is tenable that plant defences based directly or indirectly on sulphur-containing compounds such as the production of thionins (Florack and Stiekema, 1994), defensins (Broekaert et al., 1995), glucosinolates (Mansfield, 2000), glutathione (Gullner and Kömives, 2001) and sulphur-containing phytoalexins including elemental sulphur would be reduced. A higher susceptibility of *Brassica* crops against several diseases as a result of sulphur deficiency has already been observed (Schnug et al., 1995a). Scoring the infection of different oil seed rape genotypes with *Pyrenopeziza brassicae,* revealed that susceptibility of the crop to this disease was closely related to the natural sulphur supply, such that the highest infection was consistently at the site of the lowest

sulphur nutrients. Furthermore, sulphur fertilization was shown to control the infection efficiently (Schnug et al., 1995a) and the same was found to be true of grapes and the powdery mildew *Uncinula necator* (Bourbos et al., 2000). The infection of oil seed rape with *Verticillium* also decreased with increasing sulphur status of the crop (Burandt et al., 2001). In addition, a relationship has been found between sulphur supply and levels of glutathione and glucosinolates in oil seed rape plants (Schnug et al., 1995b; Haneklaus et al., 1999).

As more techniques become available it may be possible to implement the use of phytoalexins in agriculture. Elemental sulphur is still widely used as a fungicide and is an integral part of many pest management programmes because it is effective against many fungal pathogens and non-toxic to animals (Markosyan, 1970; Jolivet, 1993). It may be possible to manipulate elemental sulphur biosynthesis in order to enhance the defence of plants against fungal pathogens. This could contribute to a reduction in the use of chemical pesticides that have received increasing attention worldwide, due to the environmental pollution and ecological imbalances that they cause (Soytong et al., 2001).

Enhancing the defence of plants by altering phytoalexin production can be attempted in several ways. Firstly the regulation of existing phytoalexin systems may be altered. Over-expressing an existing gene for the rate-limiting step may increase the flux to phytoalexin synthesis, although so far attempts to do this with a sesquiterpene phytoalexin in tobacco have been unsuccessful (Chappell et al., 1995). Engineering an exaggerated response to infection, in which a plant's entire pathway for phytoalexin biosynthesis is induced more quickly and/or more strongly may enhance resistance (Essenberg, 2001) and manipulation of resveratrol synthesis is a good example of where this has worked. Four copies of an enhancer-like element from the 35S Cauliflower Mosaic Virus (CaMV) promoter were engineered upstream of the native promoter of the grapevine resveratrol synthase gene. This construct was transformed into tobacco and resulted in enhanced disease resistance (Hain, 1999). The elemental sulphur biosynthetic pathway may be up-regulated in xylem tissue or induced in leaf tissue of elemental sulphur-producing plants by introducing specific enhancers or promoters, creating transformants that are more resistant to fungal attack. However if elemental sulphur biosynthesis involves a more complex pathway, its up-regulation or

induction may only become feasible as more becomes known about the signal transduction pathways involved (Essenberg, 2001).

A second way in which elemental sulphur biosynthesis may be used to enhance the resistance of plants to pathogens is to introduce the heterologous elemental sulphur biosynthetic genes into plants that are unable to produce elemental sulphur such as species of the Rosaceae and Gramineae. Modification of phytoalexin biosynthetic pathways through the introduction of heterologous genes is becoming possible as genes controlling pathway enzymes become cloned (Essenberg, 2001). For example, many genes for biosynthesis of the isoflavonoid phytoalexins of legumes have now been cloned (Dixon and Steele, 1999) and it is hoped that the isoflavonoid biosynthetic pathway can be redirected in order to produce different enantiomers that are more toxic to invading pathogens (VanEtten et al., 1989; Delserone et al., 1992; Paiva et al., 1994). Phytoalexin engineering projects such as that involving elemental sulphur production may require the engineering of large numbers of transgenes into plants. Encouraging research has come from Chen et al. (1998) where the bombardment of embryonic rice tissues with a mixture of plasmids carrying different transgenes has resulted in plants where up to 13 different genes have integrated and as many as 10 genes were co-integrated at a single locus. However these genes then need to be expressed with co-ordinated activities. Techniques for controlling transgene expression are currently under investigation (Essenberg, 2001).

In conclusion, although there are still many technical difficulties in engineering plants to produce altered amounts of phytoalexins in response to pathogen attack, the pace of recent developments in genomics and bioinformatics provides encouragement. In order for this to be achieved for elemental sulphur the main goals for the future are firstly the elucidation of the pathway of elemental sulphur production and secondly confirmation of the role of elemental sulphur in defence. Suppression subtractive hybridisation and cDNA-AFLP analyses have already been carried out in order to isolate tomato genes up-regulated in response to *V. dahliae* that are possibly involved in elemental sulphur production (J. Howarth, unpublished data) but have so far only yielded one clone (6-5) of interest. Work is also now in progress by J. Howarth to characterise clone 6-5 that was found to be up-regulated specifically in response to *V. dahliae* in resistant tomato plants.

Perhaps another approach would be the use of a tomato microarray to detect genes up-regulated in response to *V. dahliae* infection. To the author's knowledge no complete tomato microarray is available as yet. However there are several tomato microarrays available that are enriched for genes up-regulated in response to different factors. Microarrays of genes up-regulated in response to pathogen challenge have been produced (M. Rep, Swammerdam Institute for Life Sciences, Amsterdam, The Netherlands, unpublished data). Furthermore a tomato macroarray has been produced enriched with genes up-regulated by changes in plant mineral status including sulphur deficiency (Wang et al., 2001).

Once genes of interest such as 6-5 are found, *in situ* localisation studies would be performed and mutant knock out lines identified that should suggest their contribution in resistance. Furthermore if a rapid screen for elemental sulphur deficiency were developed then random mutagenesis could be performed and plants found to be deficient in elemental sulphur production characterised in terms of disease response.

References

Adám AL, Deising H, Barna B, Gullner G, Király Z, Mendgen K (1997) Imbalances in free radical metabolism: roles in the induction of hypersensitive response and local acquired resistance of plants. *In* K Rudolph, D Burr, J Mansfield, D Stead, A Vivian, J Von Kietzell, eds, *Pseudomonas syringae* pathovars and related pathogens. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 111-121

Agrios GN (1997) Plant Pathology. Academic Press, London, UK

Aida K, Tokuyama T, Uemura T (1969) The role of cysteine desulphhydrase and cysteine synthase in the evolution of hydrogen sulphide in pantothenic acid deficient yeast. Antonie Van Leeuwenhoek **35:** Suppl: I 15-16

Aist JR, Bushnell WR (1991) Invasion of plants by powdery mildew fungi, and cellular mechanisms of resistance. *In* G Cole, H Hoch, eds, The Fungal Spore and Disease Initiation in Plants and Animals. Plenum Press, London, UK, pp 321-345

Allen JF (1977) Superoxide and photosynthetic reduction in oxygen. *In* A Michelson, J McCord, I Fridovich, eds, Superoxide and Superoxide Dismutases. Academic Press, London, UK, pp 417-436

Andersson JT, Holwitt U (1994) An advantageous reagent for the removal of elemental sulfur from environmental samples. Fresenius J Anal Chem **350:** 474-480

Aono M, Kubo A, Saji H, Tanaka K, Kondo N (1993) Enhanced tolerance to photooxidative stress of transgenic *Nicotiana tabacum* with high chloroplastic glutathione reductase activity. Plant Cell Physiol **34:** 129-135

Aono M, Saji H, Fujiyama K, Sugita M, Kondo N, Tanaka K (1995b) Decrease in activity of glutathione reductase enhances paraquat sensitivity in transgenic *Nicotiana tabacum*. Plant Physiol **107:** 645-648

Aono M, Saji H, Sakamoto A, Tanaka K, Kondo N, Tanaka K (1995a) Paraquat tolerance of transgenic *Nicotiana tabacum* with enhanced activities of glutathione reductase and superoxide dismutase. Plant Cell Physiol **36:** 1687-1691

Arieli B, Padan E, Shahak Y (1991) Sulfide-induced sulphide-quinone reductase activity in thylakoids of *Oscillatoria limnetica*. J Biol Chem **266:** 104-111

Arlorio M, Ludwig A, Boller T, Bonafonte P (1992) Inhibition of fungal growth by plant chitinases and β-1,3-glucanases: a morphological study. Protoplasma **171:** 34-43

Armah CN, Mackie AR, Roy C, Price K, Osbourn AE, Bowyer P, Ladha S (1999) The membrane-permeabilizing effect of avenacin A-1 involves the reorganization of bilayer cholesterol. Biophys J **76:** 281-290

Arneson PA, Durbin RD (1967) Hydrolysis of tomatine by *Septoria lycopersici*: a detoxification mechanism. Phytopathology **57:** 1358-1360

Arneson PA, Durbin RD (1968) Studies on the mode of action of tomatine as a fungitoxic agent. Plant Physiol **43:** 683-686

Arora DK, Hirsch PR, Kerry BR (1996) PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. Mycol Res **100:** 801-809

Ashworth LJ (1983) Aggressiveness of random and selected isolates of *Verticillium dahliae* from cotton and the quantitative relationship of internal inoculum to defoliation. Phytopathology **73:** 1292-1295

Ashworth LJ, Huisman OC, Harper DM, Stromberg LK (1979) *Verticillium* wilt disease of tomato: influence of inoculum density and root extension upon disease severity. Phytopathology **69:** 490-492

Atkinson P, Blakeman JP (1982) Seasonal occurrence of an antimicrobial flavanone, sakuranetin, associated with glands on leaves of *Ribes nigrum*. New Phytol **92:** 63-74

Ayouba A, Causse H, Van Damme EJM, Peumans WJ, Bourne Y, Cambillau C, Rouge P (1994) Interaction of plant lectins with the components of the bacterial cell wall peptidoglycan. Biochem Syst Ecol **22:** 153-159

Bachem CWB, Van der Hoeven RS, De Bruijn SM, Vreugdenhil D, Zabeau M, Visser RGF (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J **9:** 745-753

Bailey JA (1974) The relationship between symptom expression and phytoalexin concentration in hypocotyls of *Phaseolus vulgaris* infected with *Colletotrichum lindemuthianum*. Physiol Plant Pathol **4:** 477-488

Bailey JA (1982) Physiological and biochemical events associated with the expression of resistance to disease. *In* R Wood, ed, Active Defense Mechanisms in Plants. Plenum Press, London, UK, pp 39-65

Bailey JA (1987) Phytoalexins: a genetic view of their significance. *In* P Day, G Jellis, eds, Genetics and Plant Pathogenesis. Blackwell Scientific Publications, Oxford, UK, pp 233-244

Bailey JA, Carter GA, Skipp RA (1976) The use and interpretation of bioassays for fungitoxicity of phytoalexins in agar media. Physiol Plant Pathol **8:** 189-194

Bailey JA, Deverall BJ (1971) Formation and activity of phaseollin in the interaction between bean hypocotyls (*Phaseolus vulgaris*) and physiological races of *Colletotrichum lindemutianum*. Physiol Plant Pathol **1:** 435-449

Bailey JA, O'Connell RJ, Pring RJ, Nash C (1992) Infection strategies of *Colletotrichum* species. *In* J Bailey, M Jeger, eds, *Colletotrichum*: Biology, Pathology and Control. CAB International, Wallingford, UK, pp 88-120

Bailey JA, Rowell PM, Arnold GM (1980) The temporal relationship between infected cell death, phytoalexin accumulation and the inhibition of hyphal development during resistance of *Phaseolus vulgaris* to *Colletotrichum lindemuthianum*. Physiol Plant Pathol **17:** 329-339

Bailey JA, Skipp RA (1978) Toxicity of phytoalexins. Ann Appl Biol **89:** 354-358

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. Annu Rev Phytopathol **33:** 299-321

Bang SS, Deshpande SS, Han KN (1995) The oxidation of galena using *Thiobacillus ferroxidans*. Hydrometallurgy **37:** 181-192

Barbieri L, Battelli MG, Stirpe F (1993) Ribosome-inactivating proteins from plants. Biochim Biophys Acta **1154:** 237-282

Barker BTP (1929) Investigations on the fungicidal action of sulphur. Ann Res Rept Agricultural Horticulture Research Station, Long Ashton, Bristol, pp 130-148

Barker BTP, Gimingham CT, Wiltshire SP (1920) Sulphur as a fungicide. Ann Res Rept Agricultural Horticulture Research Station, Long Ashton, Bristol, pp 57-75

Barker BTP, Wallace T (1921) A new method of sulphur fumigation. Ann Res Rept Agricultural Horticulture Research Station, Long Ashton, Bristol, pp 122-124

Barroso C, Romero LC, Cejudo FJ, Vega JM, Gotor C (1999) Salt specific regulation of the cytosolic *O*-acetylserine(thiol)lyase gene from *Arabidopsis thaliana* is dependent on abscisic acid. Plant Mol Biol **40:** 729-763

Barroso C, Vega JM, Gotor C (1995) A new member of the cytosolic *O*-acetylserine(thiol)lyase gene family in *Arabidopsis thaliana*. FEBS Lett **363:** 1-5

Bartlett JK, Skoog DA (1954) Colorimetric determination of elemental sulfur in hydrocarbons. Anal Chem **26:** 1008-1011

Bartsch RG, Kamen MD (1960) Isolation and properties of two soluble heme proteins in extracts of the photoanaerobe *Chromatium*. J Bio Chem **235:** 825-831

Barz W, Welle R (1992) Biosynthesis and metabolism of isoflavones and pterocarpan phytoalexins in chickpea, soybean and phytopathogenic fungi. *In* H Stafford, R Ibrahim, eds, Phenolic Metabolism in Plants. Plenum Press, New York, USA, pp 134-164

Bateman DF, VanEtten HD, English PD, Nevins DJ, Albersheim P (1969) Susceptibility to enzymic degradation of cell walls from bean plants resistant and susceptible to *Rhizoctonia solani* Kuhn. Plant Physiol **44:** 641-648

Beauchamp RO, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA (1984) A critical review of the literature on hydrogen sulfide toxicity. Crit Rev Toxicol **13:** 25-48

Beckman CH (1987) The Nature of Wilt Diseases of Plants. APS Press, St Paul, Minnesota, USA

Beckman CH (2000) Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? Physiol Mol Plant Pathol **57:** 101-110

Beckman CH, Roberts EM (1995) On the nature and genetic basis for resistance and tolerance to fungal wilt diseases of plants. Adv Bot Res **21:** 35-77

Beckman CH, Talboys PW (1981) Anatomy of resistance. *In* M Mace, A Bell, C Beckman, eds, Fungal Wilt Diseases of Plants. Academic Press, London, UK, pp 487-521

Beckman CH, VanderMolen GE, Mueller WC, Mace ME (1976) Vascular structure and distribution of vascular pathogens in cotton. Physiol Plant Pathol **9:** 87-94

Beckman CH, Verdier PA, Mueller WC (1989) A system of defence in depth provided by vascular parenchyma cells of tomato in response to vascular infection with *Fusarium oxysporum* f. sp. *lycopersici*, race 1. Physiol Mol Plant Pathol **34:** 227-239

Beerhues L, Witte B, Hahlbrock K, Kombrink E (1990) Structure and expression of chitinase and 1,3-β-glucanase genes in potato. Plant Physiol **93:** 28

Beffa R, Meins F (1996) Pathogenesis-related functions of plant β-1,3-glucanases investigated by antisense transformation – a review. Gene **179:** 97-103

Beffa T (1993a) Metabolism of elemental sulphur (S^0) during fungal spore germination. Can J Microbiol **39:** 735-741

Beffa T (1993b) Inhibitory action of elemental sulphur (S^0) on fungal spores. Can J Microbiol **39:** 731-735

Beffa T, Pezet R, Turian G (1987) Multiple-site inhibition by colloidal elemental sulfur (S^0) of respiration by mitochondria from young dormant α spores of *Phomopsis viticola*. Physiol Plant **69:** 443-450

Behravan J, Chirakkal H, Masson A, Moir A (2000) Mututations in the *gerP* locus of *Bacillus subtilis* and *Bacillus cereus* affect access of germinants to their targets in spores. J Bacteriol **182:** 1987-1994

Bell AA, Mace ME (1981) Biochemistry and physiology of resistance. *In* M Mace, A Bell, C Beckman, eds, Fungal Wilt Diseases of Plants. Academic Press, London, UK, pp 431-486

Benhamou N (1996) Elicitor-induced plant defence pathways. Trends Plant Sci **1:** 233-240

Benhamou N, Mazau D, Grenier J, Esquerré-Tugayé MT (1991) Time-course study of the hydroxyproline-rich glycoproteins in root cells of susceptible and resistant tomato plants infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Planta **184:** 196-208

Ben-Yephet Y, Shtienberg D (1997) Effects of the host, the pathogen, the environment and their interactions, on *Fusarium* wilt in carnation. Phytoparasitica **25:** 207-216

Berbee ML, Taylor JW (1999) Fungal phylogeny. *In* R Oliver, M Schweizer, eds, Molecular Fungal Biology. Cambridge University Press, Cambridge, UK, pp 21-77

Bergmann C, Ito Y, Singer D, Albersheim P, Darvill AG, Benhamou N, Nuss L, Salvi G, Cervone F, De Lorenzo G (1994) Polygalacturonase-inhibiting protein

accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors, and fungal infection. Plant J **5:** 265-634

Bergmann L, Rennenberg H (1993) Glutathione metabolism in plants. *In* L De Kok, I Stulen, H Rennenberg, C Brunold, W Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, The Hague, The Netherlands, pp 109-123

Bhagwat B, Duncan EJ (1998) Mutation breeding of Highgate (*Musa acuminata*, AAA) for tolerance to *Fusarium oxysporum* f. sp. *cubense* using gamma irradiation. Euphytica **101:** 143-150

Bick JA, Aslund F, Chen Y, Leustek T (1998) Glutaredoxin function for the carboxyl-terminal domain of the plant-type 5'-adenylylsulfate reductase. Proc Natl Acad Sci **95:** 8404-8409

Bick JA, Leustek T (1998) Plant sulfur metabolism – the reduction of sulfate to sulfite. Curr Opin Plant Biol **1:** 240-244

Bircher U, Hohl HR (1997) Surface glycoproteins associated with appressorium formation and adhesion in *Phytophthora palmivora*. Mycol Res **101:** 769-775

Björkman R (1976) Properties and function of plant myrosinases. *In* J Vaughan, A Macleod, B Jones, eds, The Biology and Chemistry of the Cruciferae. Academic Press, London, UK, pp 191-205

Blanco MH, Johnson MG, Colbert TR, Zuber MS (1977) An inoculation technique for Stewart's wilt disease of corn. Plant Dis Reptr **61:** 413-416

Bogdanova N, Bork C, Hell R (1995) Cysteine synthesis in plants: isolation and functional characterization of a cDNA encoding a serine acetyltransferase from *Arabidopsis thaliana*. FEBS Lett **358:** 43-47

Bogdanova N, Hell R (1997) Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J **11:** 251-262

Bohlmann H, Apel K (1991) Thionins. Annu Rev Plant Physiol Plant Mol Biol **42:** 227-240

Bolchi A, Petrucco S, Tenca PL, Foroni C, Ottonello S (1999) Coordinate modulation of maize sulfate permease and ATP sulfurylase mRNAs in response to variations in sulfur nutritional status: stereospecific down-regulation by L-cysteine. Plant Mol Biol **39:** 527-537

Bolwell GP (1993) Dynamic aspects of the plant extracellular matrix. Int Rev Cytol **146:** 261-323

Bolwell GP (1996) The origin of the oxidative burst in plants. Biochem Soc T **24:** 438-442

Bolwell GP (1999) Role of active oxygen species and NO in plant defence responses. Curr Opin Plant Biol **2:** 287-294

Bolwell GP, Wojtaszek P (1997) Mechanisms for the generation of reactive oxygen species in plant defence – a broad perspective. Physiol Mol Plant Pathol **51:** 347-366

Bonas U, Van den Ackerveken G (1997) Recognition of bacterial avirulence proteins occurs inside the plant cell: a general phenomenon in resistance to bacterial diseases? Plant J **12:** 1-7

Bones A, Iversen TH (1985) Myrosin cells and myrosinase. Israel J Bot **34:** 351-376

Bones AM, Rossiter JT (1996) The myrosinase-glucosinolate system, its organisation and biochemistry. Physiol Plant **97:** 194-208

Booth C (1971) The genus *Fusarium*. Eastern Press Ltd, London, UK

Bork C, Schwenn JD, Hell R (1998) Isolation and characterization of a gene for assimilatory sulfite reductase from *Arabidopsis thaliana*. Gene **212:** 147-153

Boucher CA, Barberis PA, Trigalet AP, Demery DA (1985) Transposon mutagenesis *of Pseudomonas solanacearum*: isolation of Tn5-induced avirulent mutants. J Gen Microbiol **131:** 2449-2457

Bourbos VA, Skoudridakis MT, Barbopoulou E, Venetis K (2000) Ecological control of grape powdery mildew (*Uncinula necator*). Http://www.land-wirtschaft-mlr.baden-wuerttemberg.de/la/lvwo/kongress/SULFUR.html

Bowyer P, Clarke BR, Lunness P, Daniels MJ, Osbourn AE (1995) Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. Science **267:** 371-373

Brabban AD, Edwards C (1995) The effects of glucosinolates and their hydrolysis products on microbial growth. J Appl Bacteriol **79:** 171-177

Brantner A, Pfeiffer KP, Brantner H (1994) Applicability of diffusion methods required by the pharmacopeoias for testing antibacterial activity of natural compounds. Pharmazie **49:** 512-516

Braun EJ (1990) Colonization of resistant and susceptible maize plants by *Erwinia stewartii* strains differing in exopolysaccharide production. Physiol Mol Plant Pathol **36:** 363-379

Bringhurst RS, Hansche PE, Voth V (1968) Inheritance of *Verticillium* wilt resistance and the correlation of resistance with performance traits of the strawberry. Proc Amer Soc Hort Sci **92:** 369-375

Broadbent P, Creissen GP, Kular B, Wellburn AR, Mullineaux P (1995) Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. Plant J **8:** 247-255

Broekaert WF, Mariën W, Terras FRG, De Bolle MFC, Proost P, Van Damme J, Dillen L, Claeys M, Rees SB, Vanderleyden J, Cammue BPA (1992) Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. Biochemistry **31:** 4308-4314

Broekaert WF, Peumans WJ (1986) Lectin release from seeds of *Datura stramonium* and interference of the *Datura stramonium* lectin with bacterial motility. *In* T Bog-Hansen, E Van Driessche, eds, Lectins, Biology, Biochemistry, Clinical Biochemistry, Vol 5. Walter de Gruyter, Berlin, Germany, pp 57-65

Broekaert WF, Terras FRG, Cammue BPA, Osborn RW (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol **108:** 1353-1358

Broekaert WF, Van Parijs J, Leyns F, Joos H, Peumans WJ (1989) A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science **245:** 1100-1102

Brown AF, Juvik JA, Pataky JK (2001) Quantitative trait loci in sweet corn associated with partial resistance to Stewart's wilt, northern corn leaf blight, and common rust. Phytopathology **91:** 293-300

Brown FH, Wiles AB (1970) Reaction of certain cultivars and weeds to a pathogenic isolate of *Verticillium albo-atrum* from cotton. Plant Dis Reptr **54:** 508-512

Brown PD, Morra MJ (1997) Control of soil-borne plant pests using glucosinolate-containing plants. Adv Agron **61:** 167-231

Browne LM, Conn KL, Ayer WA, Tewari JP (1991) The camalexins: new phytoalexins produced in the leaves of *Camelina sativa* (Cruciferae). Tetrahedron **47:** 3909-3914

Brune DC (1995) Isolation and characterization of sulfur globule proteins from *Chromatium vinosum* and *Thiocapsa roseopersicina*. Arch Microbiol **163:** 391-399

Brunold C, Rennenberg H (1997) Regulation of sulfur metabolism in plants: first molecular approaches. Prog Bot **58:** 164-186

Brunold C, Suter M (1989) Localization of enzymes of assimilatory sulfate reduction in pea roots. Planta **179:** 228-234

Brust GE (1997) Differential susceptibility of pumpkins to bacterial wilt related to plant growth stage and cultivar. Crop Prot **16:** 411-414

Buchanan DH, Coombs KJ, Murphy PM (1993) A convenient method for the quantitative determination of elemental sulfur in coal by HPLC analysis of perchloroethylene extracts. Energ Fuel **7:** 219-221

Buddenhagen IW (1981) Conceptual and practical considerations when breeding for tolerance or resistance. *In* R Staples, G Toenniessen, eds, Plant Disease Control, Resistance and Susceptibility. John Wiley and Sons, Chichester, UK, pp 221-234

Bull CA (1981) Studies on the fungitoxicity, and relevance to disease resistance, of the phytoalexin, kievitone. Ph D Thesis, University of Hull, Hull, UK

Burandt P, Papenbrock J, Schmidt A, Bloem E, Haneklaus S, Schnug E (2001) Genotypical differences in total sulfur contents and cysteine desulfhydrase activities in *Brassica napus* L. Phyton **41:** 75-86

Burandt P, Schmidt A, Papenbrock J (2002) Three *O*-acetyl-L-serine(thiol)lyase isoenzymes from *Arabidopsis* catalase cysteine synthesis and cysteine desulfuration at different pH values. J Plant Physiol **159:** 111-119

Butt A, Mousley C, Morris K, Beynon J, Can C, Holub E, Greenberg JT, Buchanan-Wollaston V (1998) Differential expression of a senescence-enhanced metallothionein gene in *Arabidopsis* in response to isolates of *Peronospora parasitica* and *Pseudomonas syringae*. Plant J **16:** 209-221

Buwalda F, Stulen I, De Kok LJ, Kulper PJC (1990) Cysteine, γ-glutamyl-cysteine and glutathione contents of spinach leaves as affected by darkness and application of excess sulfur. II. Glutathione accumulation in detached leaves exposed to H_2S in the absence of light is stimulated by the supply of glycine to the petiole. Physiol Plant **80:** 196-204

Cakmak I, Marschner H (1988) Enhanced superoxide radical production in roots of zinc-deficient plants. J Exp Bot **39:** 1449-1460

Cannon DM, Palcholski ML, Ewing AG, Winograd N (1997) Molecule specific imaging of human red blood cells using ToFSIMS. *In* G Gillen, R Lareau, J Bennett, F Stevie, eds, Secondary Ion Mass Spectrometry (SIMSXI). John Wiley and Sons, Chichester, UK, pp 93-96

Cartwright DW, Langcake P, Ride JP (1980) Phytoalexin production in rice and its enhancement by a dichlorocyclopropane fungicide. Physiol Plant Pathol **17:** 259-267

Carver TLW, Ingerson SM (1987) Responses of *Erysiphe graminis* to contact with artificial and host surfaces. Physiol Mol Plant Pathol **30:** 359-372

Chan CW, Suzuki I (1993) Quantitative extraction and determination of elemental sulfur and stoichiometric oxidation of sulfide to elemental sulfur by *Thiobacillus thiooxidans*. Can J Microbiol **39:** 1166-1168

Chappell J, Wolf F, Proulx J, Cuellar R, Saunders C (1995) Is the reaction catalyzed by 3-hydroxy-3-methyl-glutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants? Plant Physiol **109:** 1337-1343

Charkowski AO, Alfano JR, Preston G, Yuan J, He SY, Collmer A (1998) The *Pseudomonas syringae* pv. *tomato* HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. J Bacteriol **180:** 5211-5217

Charni N, Perissol C, Le Petit J, Rugani N (2000) Production and characterization of monoclonal antibodies against vegetative cells of *Bacillus cereus*. Appl Environ Microbiol **66:** 2278-2281

Chen L, Marmey P, Taylor NJ, Brizard JP, Espinoza C, D'Cruz P, Huet H, Zhang S, De Kochko A, Beachy RN, Fauquet CM (1998) Expression and inheritance of multiple transgenes in rice plants. Nat Biotechnol **16:** 1060-1061

Chen S, Andreasson E (2001) Update on glucosinolate metabolism and transport. Plant Physiol Biochem **39:** 743-758

Christen AA, Peaden RN, Harris GP, Heale JB (1983) Virulence of North American and European isolates of *Verticillium albo-atrum* on alfalfa cultivars. Phytopathology **73:** 1051-1054

Ciuffetti LM, Weltring KM, Turgeon BG, Yoder OC, VanEtten HD (1988) Transformation of *Nectria haematococca* with a gene for pisatin demethylating activity, and the role of pisatin detoxification in virulence. J Cell Biochem **12C:** 278

Clark PD, Lesage KL (1989) Quantitative determination of elemental sulfur in hydrocarbons, soils, and other materials. J Chromatogr Sci **27:** 259-261

Clarkson DT, Hawkesford MJ, Davidian JC (1993) Membrane and long-distance transport of sulfate. *In* L De Kok, I Stulen, H Rennenberg, C Brunold, W Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, The Hague, The Netherlands, pp 3-19

Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J **18:** 3325-3333

Cobbett CS (2000) Phytochelatin biosynthesis and function in heavy-metal detoxification. Curr Opin Plant Biol **3:** 211-216

Cobbett CS (2001) Heavy metal detoxification in plants: phytochelatin biosynthesis and function. IUBMB Life **51:** 183-188

Cobbett CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. Plant J **16:** 73-78

Cole MD (1994) Key antifungal, antibacterial and anti-insect assays – a critical review. Biochem Syst Ecol **22:** 837-856

Colliver TL, Brummel C, Palcholski ML, Swanek FD, Ewing AG, Winograd N (1997) Atomic and molecular imaging at the single-cell level with ToFSIMS. Anal Chem **69:** 2225-2231

Conway WS, MacHardy WE (1978) Distribution and growth *of Fusarium oxysporum* f. sp. *lycopersici* Race 1 or Race 2 within tomato plants resistant or susceptible to wilt. Phytopathology **68:** 938-942

Cooper RM (2000) *Verticillium*-host interactions: past achievements and future molecular prospects: an overview. *In* E Tjamos, R Rowe, J Heale, D Fravel, eds, Advances in *Verticillium* Research and Disease Management. APS Press, St Paul, Minnesota, USA, pp 144-150

Cooper RM, Resende MLV, Flood J, Rowan MG, Beale MH, Potter U (1996) Detection and cellular localization of elemental sulphur in disease resistant genotypes of *Theobroma cacao*. Nature **379:** 159-162

Cooper RM, Wood RKS (1980) Cell wall degrading enzymes of vascular wilt fungi. III. Possible involvement of endo-pectin lyase in *Verticillium* wilt of tomato. Physiol Plant Pathol **16:** 285-300

Cotton Seed Distributors (2001-2002) Variety Guide. Cotton Seed Distributers Ltd., Dalby, Queensland, Australia

Courtney H, Lambeth VN (1977) Glycoalkaloid content of mature green fruit of *Lycopersicon* species. Science **12:** 550-551

Creissen G, Edwards EA, Enard C, Wellburn A, Mullineaux P (1992) Molecular characterization of glutathione reductase cDNAs from pea (*Pisum sativum* L.). Plant J **2:** 129-131

Creissen G, Mullineaux P (1995) Cloning and characterization of glutathione reductase cDNAs and identification of two genes encoding the tobacco enzyme. Planta **197:** 422-425

Crombie L, Crombie WML, Whiting DA (1984) Isolation of avenacins A-1, A-2, B-1, B-2 from oat roots: structures of their "aglycones", the avenestergenins. J Chem Soc Chem Commun **4:** 244-246

Crombie WML, Crombie L (1986) Distribution of avenacins A-1, A-2, B-1 and B-2 in oat roots: their fungicidal activity towards "take-all" fungus. Phytochemistry **25:** 2069-2073

Crombie WML, Crombie L, Green JB, Lucas JA (1986) Pathogenicity of "take all" fungus to oats: its relationship to the concentration and detoxification of the four avenacins. Phytochemistry **25:** 2075-2083

Cross H, Brick MA, Schwartz HF, Panella LW, Byrne PF (2000) Inheritance of resistance to *Fusarium* wilt in two common bean races. Crop Sci **40:** 954-958

Cruickshank IAM (1962) Studies on phytoalexins. IV. The antimicrobial spectrum of pisatin. Aust J Biol Sci **15:** 147-159

Cruickshank IAM, Perrin DR (1961) Studies on phytoalexins III. The isolation assay and general properties of a phytoalexin from *Pisum sativum* L. Aust J Biol Sci **14:** 336-348

Cruickshank IAM, Perrin DR (1963) Studies on phytoalexins. VI. Pisatin: the effect of some factors on its formation in *Pisum sativum* L., and the significance of pisatin in disease resistance. Aust J Biol Sci **16:** 111-128

Cruickshank IAM, Perrin DR (1971) Studies on phytoalexins. XI. The induction, antimicrobial spectrum and chemical assay of phaseollin. Phytopathol Z **70:** 209-229

Crute IR, De Wit PJGM, Wade M (1985) Mechanisms by which genetically controlled resistance and virulence influence host colonisation by fungal and bacterial parasites. *In* R Fraser, ed, Mechanisms of Resistance to Plant Diseases. Martinus Nijhoff/Dr W. Junk Publishers, Lancaster, UK, pp 197-284

Cusanovich MA, Meyer TE, Bartsch RG (1991) Flavocytochrome *c*. *In* F Muller, ed, Chemistry and Biochemistry of Flavoenzymes. CRC Press, Boca Raton, Florida, USA, pp 377-399

Dackman C, Bååth E (1989) Effect of temperature and time on growth of *Verticillium chlamydosporium* and *V. suchlasporium* and infection of *Heterodera avenae* eggs. Mycologia **81:** 161-163

Dai GH, Nicole M, Andary C, Martinez C, Bresson E, Boher B, Daniel JF, Geiger JP (1996) Flavonoids accumulate in cell walls, middle lamellae and callose-rich papillae during an incompatible interaction between *Xanthomonas campestris* pv. *malvacearum* and cotton. Physiol Mol Plant Pathol **49:** 285-306

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature **411:** 826-833

Datko AH, Mudd SH (1982) Methionine biosynthesis in *Lemna* – inhibitor studies. Plant Physiol **69:** 1070-1076

De Kok LJ, Stulen I (1993) Role of glutathione in plants under oxidative stress. *In* L De Kok, I Stulen, H Rennenberg, C Brunold, W Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, The Hague, The Netherlands, pp 125-138

De Lorenzo G, Cervone F, Bellincampi D, Caprari C, Clark AJ, Desidero A, Devoto A, Forrest R, Leckie F, Nuss L, Salvi G (1994) Polygalacturonase, PGIP and oligogalacturonides in cell-cell communication. Biochem Soc T **22:** 394-397

De Lorenzo G, D'Ovidio R, Cervone F (2001) The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu Rev Phytopathol **39:** 313-335

Del Sorbo GD, Andrade AC, Van Nistelroy JGM, Van Ken JAL, Balzi E, De Waard MA (1997) Multi-drug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters. Mol Gen Genet **254:** 417-426

Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature **394:** 585-588

Delserone LM, Matthews DE, VanEtten HD (1992) Differential toxicity of enantiomers of maackiain and pisatin to phytopathogenic fungi. Phytochemistry **31:** 3813-3819

Delserone LM, McCluskey K, Matthews DE, VanEtten HD (1999) Pisatin demethylation by fungal pathogens and nonpathogens of pea: association with pisatin tolerance and virulence. Physiol Mol Plant Pathol **55:** 317-326

Denny TP, Matthews PS, VanEtten HD (1987) A possible mechanism of nondegradative tolerance of pisatin in *Nectria haematococca* MP VI. Physiol Mol Plant Pathol **30:** 93-107

Denny TP, VanEtten HD (1983) Tolerance of *Nectria haematococca* MP VI to the phytoalexin pisatin in the absence of detoxification. J Gen Microbiol **129:** 2893-2901

Desjardins AE, Gardner HW (1991) Virulence of *Gibberella pulicaris* on potato tubers and its relationship to a gene for rishitin metabolism. Phytopathology **81:** 429-435

Desjardins AE, VanEtten HD (1986) Partial purification of pisatin demethylase, a cytochrome P-450 from the pathogenic fungus *Nectria haematococca*. Arch Microbiol **144:** 84-90

Deverall BJ, Rogers PM (1972) The effect of pH and composition of test solutions on the inhibitory activity of wyerone acid towards germination of fungal spores. Ann Appl Biol **72:** 301-305

Devey ME, Rosielle AA (1986) Relationship between field and greenhouse ratings for tolerance to *Verticillium* wilt on cotton. Crop Sci **26:** 1-5

Dhindsa RS (1991) Drought stress, enzymes of glutathione metabolism, oxidation injury, and protein synthesis in *Tortula ruralis*. Plant Physiol **95:** 648-651

Dietz KJ, Baier M, Krämer U (1999) Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. *In* M Prasad, J Hagemeyer, eds, Heavy Metal Stress in Plants: From Molecules to Ecosystems. Springer-Verlag, Berlin, Germany, pp 73-97

Diwan N, Fluhr R, Eshed Y, Zamir D, Tanksley SD (1999) Mapping of *Ve* in tomato: a gene conferring resistance to the broad-spectrum pathogen *Verticillium dahliae* race 1. Theor Appl Genet **98:** 315-319

Dixon RA (2001) Natural products and plant disease resistance. Nature **411:** 843-847

Dixon RA, Steele CL (1999) Flavonoids and isoflavonoids - a gold mine for metabolic engineering. Trends Plant Sci **4:** 394-400

Doke N, Miura Y, Leandro MS, Kawakita K (1994) Involvement of superoxide in signal transduction: responses to attack by pathogens, physical and chemical shocks, and UV irridiation. *In* C Foyer, P Mullineaux, eds, Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, Florida, USA, pp 177-197

Dong X (1998) SA, JA, ethylene, and disease resistance in plants. Curr Opin Plant Biol **1:** 316-323

Dong X (2001) Genetic dissection of systemic acquired resistance. Curr Opin Plant Biol **4:** 309-314

Doran WL (1917) Controlling snapdragon rust. Value of copper and sulphur. Florist's Exchange **43:** 501

Doran WL (1922) Laboratory studies of the toxicity of some sulphur fungicides. New Hampshire State Agricultural Experiment Station, Tech Bull **19:** 3-11

Dron M, Clouse SD, Dixon RA, Lawton MA (1988) Glutathione and fungal elicitor regulation of a plant defense gene promoter in electroporated protoplasts. Proc Natl Acad Sci **85:** 6738-6742

Droux M, Ravanel S, Douce R (1995) Methionine biosynthesis in higher plants. II. Purification and characterization of cystathionine β-lyase from spinach chloroplasts. Arch Biochem Biophys **316:** 585-595

Droux M, Ruffet ML, Douce R, Job D (1998) Interactions between serine acetyltransferase and *O*-acetylserine (thiol) lyase in higher plants. Structural and kinetic properties of the free and bound enzymes. Eur J Biochem **255:** 235-245

Düring K (1993) Can lysozymes mediate antibacterial resistance in plants? Plant Mol Biol **23:** 209-214

Durrands PK, Cooper RM (1988) The role of pectinases in vascular wilt disease as determined by defined mutants of *Verticillium albo-atrum*. Physiol Mol Plant Pathol **32:** 363-371

Ebel J, Cosio EG (1994) Elicitors of plant defense responses. Int Rev Cytol **148:** 1-36

Ebel J, Mithöfer A (1998) Early events in the elicitation of plant defence. Planta **206:** 335-348

Ebel J, Scheel D (1997) Signals in host-parasite interactions. *In* G Carroll, P Tudzynski, eds, The Mycota; Vol V, Plant Relationships, Part A. Springer-Verlag, Berlin, Germany, pp 85-105

Edwards EA, Rawsthorne S, Mullineaux PM (1990) Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (*Pisum sativum* L.). Planta **180:** 278-284

Edwards R, Blount JW, Dixon RA (1991) Glutathione and elicitation of the phytoalexin response in legume cell cultures. Planta **184:** 403-409

Edwards R, Dixon DP, Walbot V (2000) Plant glutathione *S*-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci **5:** 193-198

Eichel J, González JC, Hotze M, Matthews RG, Schröder J (1995) Vitamin-B_{12}-independent methionine synthase from a higher plant (*Catharanthus roseus*). Molecular characterization, regulation, heterologous expression, and enzyme properties. Eur J Biochem **230:** 1053-1058

El-Zahaby HM, Gullner G, Király Z (1995) Effects of powdery mildew infection of barley on the ascorbate – glutathione cycle and other antioxidants in different host-pathogen interactions. Phytopathology **85:** 1225-1230

Enkerli J, Bhatt G, Covert SF (1998) Maackiain detoxification contributes to the virulence of *Nectria haematococca* MPVI on chickpea. Mol Plant-Microbe Interact **11:** 317-326

Enkerli K, Hahn MG, Mims CW (1997) Ultrastructure of compatible and incompatible interactions of soybean roots infected with the plant pathogenic Oomycete *Phytophthora sojae*. Can J Bot **75:** 1493-1508

Essenberg M (2001) Prospects for strengthening plant defenses through phytoalexin engineering. Physiol Mol Plant Pathol **59:** 71-81

Essenberg M, Pierce ML, Cover EC, Hamilton B, Richardson PE, Scholes VE (1992) A method for determining phytoalexin concentrations in fluorescent, hypersensitively necrotic cells in cotton leaves. Physiol Mol Plant Pathol **41:** 101-109

Ettlinger MG, Lundeen AJ (1956) The structures of sinigrin and sinalbin; an enzymatic rearrangement. J Am Chem Soc **78:** 4172-4173

Evans G (1971) Influence of weed hosts on the ecology of *Verticillium dahliae* in newly cultivated areas of the Namoi valley, New South Wales. Ann Appl Biol **67:** 169-175

Eyre JV, Salmon ES (1916) The fungicidal properties of certain spray-fluids. J Agr Sci **7:** 473-507

Fahey JW, Zalcmann AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry **56:** 5-51

Falisse A, Bodson B (1987) Use of elemental sulphur in cereal crops. *In* Syndicat Français du Soufre, ed, Proceedings of the International Symposium for Elemental Sulphur in Agriculture, Nice, France, pp 427-432

Fall AL, Byrne PF, Jung G, Coyne DP, Brick MA, Schwartz HF (2001) Detection and mapping of a major locus for *Fusarium* wilt resistance in common bean. Crop Sci **41:** 1494-1498

Farago S, Brunold C (1994) Regulation of thiol contents in maize roots by intermediates and effectors of glutathione synthesis. J Plant Physiol **144:** 433-437

Farkas V (1979) Biosynthesis of cell walls of fungi. Microbiol Rev **43:** 117-144

Ferreras JM, Iglesias R, Barbieri L, Alegre C, Bolognesi A, Rojo MA, Carbajales ML, Escarmis C, Girbes T (1995) Effects and molecular action of ribosome-inactivating proteins on ribosomes from *Streptomyces lividans*. Biochim Biophys Acta **1243:** 85-93

Feys BJ, Parker JE (2000) Interplay of signaling pathways in plant disease resistance. Trends Genet **16:** 449-455

Filippone MP, Diaz-Ricci JC, Castagnaro AP, Farías RN (2001) Effect of fragarin on the cytoplasmic membrane of the phytopathogen *Clavibacter michiganensis*. Mol Plant-Microbe Interact **14:** 925-928

Finney DJ (1964) Probit Analysis. Cambridge University Press, Cambridge, UK

Fiola JA, Swartz HJ (1994) Inheritance of tolerance to *Verticillium albo-atrum* in raspberry. HortScience **29:** 1071-1073

Fischer U, Trüper HG (1979) Some properties of cytochrome *c* and other hemoproteins of *Thiocapsa roseopersicina*. Curr Microbiol **3:** 41-44

Flor HH (1956) The complementary genetic systems in flax and flax rust. Adv Genet **8:** 29-54

Flor HH (1971) Current status of the gene-for-gene concept. Annu Rev Phytopathol **9:** 275-296

Florack DEA, Stiekema WJ (1994) Thionins: properties, possible biological roles and mechanisms of action. Plant Mol Biol **26:** 25-37

Fluhr R, Mattoo AK (1996) Ethylene-biosynthesis and perception. Crit Rev Plant Sci **15:** 479-523

Fock I, Collonnier C, Purwito A, Luisetti J, Souvannavong V, Vedel F, Servaes A, Ambroise A, Kodja H, Ducreux G, Sihachakr D (2000) Resistance to bacterial wilt in somatic hybrids between *Solanum tuberosum* and *Solanum phureja.* Plant Sci **160:** 165-176

Foo HL, Grønning LM, Goodenough L, Bones AM, Danielsen BE, Whiting DA, Rossiter JT (2000) Purification and characterisation of epithiospecifier protein from *Brassica napus*: enzymic intramolecular sulphur addition within alkenyl thiohydroximates derived from alkenyl glucosinolate hydrolysis. FEBS Lett **468:** 243-246

Foreman FW (1910) The fungicidal properties of liver of sulphur. J Agr Sci **3:** 400-416

Forsyth W (1802) A Treatise on the Culture and Management of Fruit Trees. Nichols and Son, London, UK

Foster RE, Walker JC (1947) Predisposition of tomato to Fusarium wilt. J Agr Res **74:** 195-185

Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. Physiol Plant **92:** 696-717

Foyer CH, Lopez-Delgado H, Dat JF, Scott IM (1997) Hydrogen peroxide- and glutathione-assoicated mechanisms of acclimatory stress tolerance and signalling. Physiol Plant **100:** 241-254

Foyer CH, Rennenberg H (2000) Regulation of glutathione synthesis and its role in abiotic and biotic stress defence. *In* C Brunold, H Rennenberg, L De Kok, I Stulen, J Davidian, eds, Sulfur Nutrition and Assimilation in Higher Plants. Paul Haupt, Bern, Switzerland, pp 127-153

Foyer CH, Souriau N, Perret S, Lelandais M, Kunert KJ, Pruvost C, Jouanin L (1995) Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. Plant Physiol **109:** 1047-1057

Frendo P, Jiménez MJH, Mathieu C, Duret L, Gallesi D, Van de Sype G, Hérouart D, Puppo A (2001) A *Medicago truncatula* homoglutathione synthetase is derived from glutathione synthetase by gene duplication. Plant Physiol **126:** 1706-1715

Friedrich CG (1998) Physiology and genetics of sulfur-oxidizing bacteria. Adv Microb Physiol **39:** 235-289

Fukumori Y, Yamanaka T (1979) Flavocytochrome *c* of *Chromatium vinosum*. J Biochem **85:** 1405-1414

Galet P (1996) Grape Diseases. Oneo Plurimedia Publishers, Chaintre, France, pp 253

Galili G (1995) Regulation of lysine and threonine synthesis. Plant Cell **7:** 899-906

Gao H, Beckman CH, Mueller WC (1995) The rate of vascular colonization as a measure of the genotypic interaction between various cultivars of tomato and various formae or races of *Fusarium oxysporum*. Physiol Mol Plant Pathol **46:** 29-43

Garber RH, Houston BR (1966) Penetration and development of *Verticillium albo-atrum* in the cotton plant. Phytopathology **56:** 993-1118

Garber RH, Houston BR (1967) Nature of *Verticillium* wilt resistance in cotton. Phytopathology **57:** 885-888

Garcia-Perez MD, Egea C, Candela ME (1998) Defence response of pepper (*Capsicum annuum*) suspension cells to *Phytophthora capsici*. Physiol Plant **103:** 527-533

Gardner HW, Desjardins AE, McCormick SP, Weisleder D (1994) Detoxification of the potato phytoalexin rishitin by *Gibberella pulicaris*. Phytochemistry **37:** 1001-1005

Gaye MM, Ormrod DJ, Seywerd FM, Odermatt WJ (1991) Occurrence of fusarium yellows on celery in southwestern British Columbia and evaluation of cultivars for disease tolerance. Can J Plant Pathol **13:** 88-92

Gentile IA, Matta A (1976) Preliminary observations on the presence of rishitin in *Fusarium* infected tomato plants. Phytopathol Mediterr **15:** 65-66

George HL, Hirschi KD, VanEtten HD (1998) Biochemical properties of the products of cytochrome P450 genes (PDA) encoding pisatin demethylase activity in *Nectria haematococca*. Arch Microbiol **170:** 147-154

Giamoustaris A, Mithen R (1997) Glucosinolates and disease resistance in oilseed rape (*Brassica napus* ssp. *oleifera*). Plant Pathol **46:** 271-275

Gibbins LN, Wright DSC (1968) *Verticillium* wilt of tobacco. VI. The influence of roots and stems on *Verticillium* wilt symptoms in tobacco. New Zeal J Agr Res **11:** 789-796

Gilchrist DG (1998) Programmed cell death in plant disease: the purpose and promise of cellular suicide. Annu Rev Phytopathol **36:** 393-414

Gill MS, MacLeod AJ, Moreau M (1984) Volatile components of cocoa with particular reference to glucosinolate products. Phytochemistry **23:** 1937-1942

Giovanelli J, Mudd SH, Datko AH (1985) Quantitative-analysis of pathways of methionine metabolism and their regulation in *Lemna*. Plant Physiol **77:** 450-455

Girbés T, De Torre C, Iglesias R, Ferreras JM (1996) RIP for viruses. Nature **379:** 777-778

Glazebrook J, Ausubel FM (1994) Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc Natl Acad Sci **91:** 8955-8959

Glazebrook J, Rogers EE, Ausubel FM (1996) Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. Genetics **143:** 973-982

Glazebrook J, Zook M, Mert F, Kagan I, Rogers EE, Crute IR, Holub EB, Hammerschmidt R, Ausubel FM (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. Genetics **146:** 381-392

Glazener JA, Orlandi EW, Baker CJ (1996) The active oxygen response of cell suspensions to incompatible bacteria is not sufficient to cause hypersensitive cell death. Plant Physiol **110:** 759-763

Goettel MS, Inglis GD (1997) Fungi: Hyphomycetes. *In* L Lacey, ed, Manual of Techniques in Insect Pathology. Academic Press, London, UK, pp 213-249

Goodman RN, Novacky AJ (1994) The Hypersensitive Reaction in Plants to Pathogens. APS Press, St Paul, Minnesota, USA

Govrin EM, Levine A (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr Biol **10:** 751-757

Graham LS, Sticklen MB (1994) Plant chitinases. Can J Bot **72:** 1057-1083

Grant BR, Irving HR, Radda M (1985) The effect of pectin and related compounds on encystment and germination of *Phytophthora palmivora* zoospores. J Gen Microbiol **131:** 669-676

Grant JJ, Loake GJ (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol **124:** 21-29

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J (2000) The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J **23:** 441-450

Grant M, Mansfield J (1999) Early events in host-pathogen interactions. Curr Opin Plant Biol **2:** 312-319

Gray GO, Knaff DB (1982) The role of a cytochrome *c*-552 in the oxidation of sulfide in *Chromatium vinosum*. Biochim Biophys Acta **680:** 290-296

Grayer RJ, Harborne JB (1994) A survey of antifungal compounds from higher plants, 1982-1993. Phytochemistry **37:** 19-42

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science **281:** 1309-1312

Greenhalgh JR, Mitchell ND (1976) The involvement of flavour volatiles in the resistance to downy mildew of wild and cultivated forms of *Brassica oleracea*. New Phytol **77:** 391-398

Greenwood NU, Earnshaw A (1997) Chemistry of the Elements. Butterworth-Heinemann, Oxford, UK

Griffith JM, Iser JR, Grant BR (1988) Calcium control of differentiation in *Phytophthora palmivora*. Arch Microbiol **149:** 565-571

Griffiths DA (1982) Structure of *Verticillium nigrescens* and *V. nubilum* chlamydospores. Trans Br Mycol Soc **78:** 141-145

Grill E, Löffler S, Winnacker EL, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proc Natl Acad Sci **86:** 6838-6842

Grimault V, Prior P (1993) Bacterial wilt resistance in tomato associated with tolerance of vascular tissues to *Pseudomonas solanacearum*. Plant Pathol **42:** 589-594

Grob K, Matile P (1979) Vacuolar location of glucosinolates in horseradish root cells. Plant Sci Lett **14:** 327-335

Grossman A, Takahashi H (2001) Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. Annu Rev Plant Physiol Plant Mol Biol **52:** 163-210

Gu Q, Kawata EE, Morse MJ, Wu HM, Cheung AY (1992) A flower-specific cDNA encoding a novel thionin in tobacco. Mol Gen Genet **234:** 89-96

Guerbette F, Grosbois M, Jolliot-Croquin A, Kader JC, Zachowski A (1999) Lipid-transfer proteins from plants: structure and binding properties. Mol Cell Biochem **192:** 157-161

Gullner G, Kömives T (2001) The role of glutathione and glutathione-related enzymes in plant-pathogen interactions. *In* D Grill, M Tausz, L De Kok, eds, Significance of Glutathione to Plant Adaption to the Environment. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 207-239

Guo ZJ, Nakagawara S, Sumitani K, Ohta Y (1993) Effect of intracellular glutathione level on the production of 6-methoxymellein in cultured carrot (*Daucus carota*) cells. Plant Physiol **102:** 45-51

Gutierrez-Marcos JF, Roberts MA, Campbell EI, Wray JL (1996) Three members of a novel small gene-family from *Arabidopsis thaliana* able to complement functionally an *Escherichia coli* mutant defective in PAPS reductase activity encode proteins with a thioredoxin-like domain and "APS reductase" activity. Proc Natl Acad Sci **93:** 13377-13382

Ha SB, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. Plant Cell **11:** 1153-1163

Hahlbrock K, Grisebach H (1979) Enzymic controls in the biosynthesis of lignin and flavonoids. Annu Rev Plant Physiol **30:** 105-130

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol **40:** 347-369

Hahlbrock K, Scheel D, Logemenn E, Nürnberger T, Parniske M, Reinold S, Sacks WR, Schmelzer E (1995) Oligopeptide elicitor-mediated defense gene activation in cultured parsley cells. Proc Natl Acad Sci **92:** 4150-4157

Hahn MG, Bonhoff A, Grisebach H (1985) Quantitative localization of the phytoalexin glyceollin I in relation to fungal hyphae in soybean roots infected with *Phytophthora megasperma* f. sp. *glycinea*. Plant Physiol **77:** 591-601

Hain R (1999) Modification of plant secondary metabolism by foreign phytoalexin genes. Special Publication – Royal Society of Chemistry **233:** 190-203

Halkier BA, Du L (1997) The biosynthesis of glucosinolates. Trends Plant Sci **2:** 425-431

Hall AA, Bindslev L, Rouster J, Rasmussen SW, Oliver RP, Gurr SJ (1999) Involvement of cAMP and protein kinase A in conidial differentiation by *Erysiphe graminis* f. sp. *hordei*. Mol Plant-Microbe Interact **12:** 960-968

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot **53:** 1-11

Halliwell B, Gutteridge JMC (1989) Free Radicals in Biology and Medicine. Clarendon Press, Oxford, UK

Hammerschmidt R (1999) Phytoalexins: what have we learned after 60 years? Annu Rev Phytopathol **37:** 285-306

Haneklaus S, Paulsen HM, Gupta AK, Bloem E, Schnug E (1999) Influence of sulfur fertilization on yield and quality of oilseed rape and mustard. Proc 10th Int Rapeseed Congress, Canberra, Australia, pp 26-29

Harada E, Choi YE, Tsuchisaka A, Obata H, Sano H (2001) Transgenic tobacco plants expressing a rice cysteine synthase gene are tolerant to toxic levels of cadmium. J Plant Physiol **158:** 655-661

Harborne JB, Williams CA (1994) Recent advances in the chemosystematics of the monocotyledons. Phytochemistry **37:** 3-18

Hargreaves JA, Bailey JA (1978) Phytoalexin production by hypocotyls of *Phaseolus vulgaris* in response to constitutive metabolites released by damaged bean cells. Physiol Plant Pathol **13:** 89-100

Hargreaves JA, Mansfield JW, Rossall S (1977) Changes in phytoalexin concentration in tissues of the broad bean plant (*Vicia faba* L.) following inoculation with species of *Botrytis*. Physiol Plant Pathol **11:** 227-242

Harris DC, Yang JR (1996) The relationship between the amount of *Vericillium dahliae* in soil and the incidence of strawberry wilt as a basis for disease risk prediction. Plant Pathol **45:** 106-114

Harris JE, Dennis C (1977) The effect of post-infectional potato tuber metabolites and surfactants on zoospores of Oomycetes. Physiol Plant Pathol **9:** 155-165

Hartill WFT, Sutton PG (1980) Inhibition of germination of *Mycosphaerella brassicicola* ascospores on young cabbage and cauliflower leaves. Ann Appl Biol **96:** 153-161

Hashem FA, Saleh MM (1999) Antimicrobial components of some cruciferae plants (*Diplotaxis harra* Forsk. and *Erucaria microcarpa* Bois.). Phytother Res **13:** 329-332

Hatzfeld Y, Cathala N, Grignon C, Davidian JC (1998) Effect of ATP sulfurylase overexpression in Bright Yellow 2 tobacco cells. Plant Physiol **116:** 1307-1313

Hatzfeld Y, Lee S, Lee M, Leustek T, Saito K (2000) Functional characterization of a gene encoding a fourth ATP sulfurylase isoform from *Arabidosis thaliana*. Gene **248:** 51-58

Hazeu W, Batenburg-Van der Vegte WH, Bos P, Van der Pas RK, Kuenen JG (1988) The production and utilization of intermediary elemental sulfur during the oxidation of reduced sulfur compounds by *Thiobacillus ferrooxidans*. Arch Microbiol **150:** 574-579

He SY (1996) Elicitation of plant hypersensitive response by bacteria. Plant Physiol **112:** 865-869

Heale JB (1988) *Verticillium* spp., the cause of vascular wilts in many species. Adv Plant Pathol **6:** 291-312

Heale JB (2000) Diversification and speciation in *Verticillium* – an overview. *In* E Tjamos, R Rowe, J Heale, D Fravel, eds, Advances in *Verticillium* Research and Disease Management. APS Press, St Paul, Minnesota, USA, pp 1-14

Heath MC (1998a) Apoptosis, programmed cell death and the hypersensitive response. Eur J Plant Pathol **104:** 117-124

Heath MC (1998b) Involvement of reactive oxygen species in the response of resistant (hypersensitive) or susceptible cowpeas to the cowpea rust fungus. New Phytol **138:** 251-263

Heath MC (2000a) Nonhost resistance and nonspecific plant defenses. Curr Opin Plant Biol **3:** 315-319

Heath MC (2000b) Multigenic disease resistance and the basis of host genotype specificity. Physiol Mol Plant Pathol **57:** 189-190

Heath MC (2000c) Hypersensitive response-related death. Plant Mol Biol **44:** 321-334

Heath MC, Nimchuk ZL, Xu H (1997) Plant nuclear migrations as indicators of critical interactions between resistant or susceptible cowpea epidermal cells and invasion hyphae of the cowpea rust fungus. New Phytol **135:** 689-700

Heiss S, Schäfer HJ, Haag-Kerwer A, Rausch T (1999) Cloning sulfur assimilation genes of *Brassica juncea* L.: cadmium differentially affects the expression of a putative low-affinity sulfate transporter and isoforms of ATP sulfurylase and APS reductase. Plant Mol Biol **39:** 847-857

Heitz T, Segond S, Kauffmann S, Geoffroy P, Prasad V, Brunner F, Fritig B, Legrand M (1994) Molecular characterization of a novel tobacco pathogenesis-related (PR) protein: a new plant chitinase/lysozyme. Mol Gen Genet **245:** 246-254

Hell R (1997) Molecular physiology of plant sulfur metabolism. Planta **202:** 138-148

Hell R, Bergmann L (1988) Glutathione synthetase in tobacco suspension cultures: catalytic properties and localization. Physiol Plant **72:** 70-76

Hell R, Bergmann L (1990) γ-glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. Planta **180:** 603-612

Hell R, Bork C, Bogdanova N, Frolov I, Hauschild R (1994) Isolation and characterization of two cDNAs encoding for compartment specific isoforms of *O*-acetylserine(thiol)lyase from *Arabidopsis thaliana*. FEBS Lett **351:** 257-262

Henshaw PF, Bewtra JK, Biswas N (1997) Extraction of elemental sulfur from an aqueous suspension for analysis by high-performance liquid chromatography. Anal Chem **69:** 3119-3123

Herschbach C, Rennenberg H (1991) Influence of glutathione (GSH) on sulfate influx, xylem loading and exudation in excised tobacco roots. J Exp Bot **42:** 1021-1029

Herschbach C, Rennenberg H (1994) Influence of glutathione (GSH) on net uptake of sulphate and sulphate transport in tobacco plants. J Exp Bot **45:** 1069-1076

Hesse H, Hoefgen R (1998) Isolation of cDNAs encoding cytosolic (accession no. AF044172) and plastidic (accession no. AF044173) cysteine synthase isoforms from *Solanum tuberosum* (PGR98-057). Plant Physiol **116:** 1604

Hesse H, Lipke J, Altmann T, Hofgen R (1999) Molecular cloning and expression analysis of mitochondrial and plastidic isoforms of cysteine synthase (*O*-acetylserine(thiol)lyase) from *Arabidopsis thaliana*. Amino Acids **16:** 113-131

Hill MK, Lyon KJ, Lyon BR (1999) Identification of disease response genes expressed in *Gossypium hirsutum* upon infection with the wilt pathogen *Verticillium dahliae*. Plant Mol Biol **40:** 289-296

Hinsch M, Staskawicz B (1996) Identification of a new *Arabidopsis* disease resistance locus, *RPS4*, and cloning of the corresponding avirulence gene, *avrRps4*, from *Pseudomonas syringae* pv. *pisi*. Mol Plant-Microbe Interact **9:** 55-61

Hislop EC, Clifford DR (1975) Bio-assay of agricultural and horticultural fungicides. *In* R Board, D Lovelock, eds, Some Methods for Microbiological Assay. Academic Press, London, UK, pp 169-189

Höglund AS, Lenman M, Falk A, Rask L (1991) Distribution of myrosinase in rapeseed tissues. Plant Physiol **95:** 213-221

Höglund AS, Lenman M, Rask L (1992) Myrosinase is localized to the interior of myrosin grains and is not associated to the surrounding tonoplast membrane. Plant Sci **85:** 165-170

Holliday P (1989) A Dictionary of Plant Pathology. Cambridge University Press, London, UK

Holliday R (1974) *Ustilago maydis. In* R King, ed, Handbook of Genetics. Plenum Press, New York, USA, pp 575-595

Horsfall JG (1956) Principles of Fungicidal Action. Chronica Botanica Company, Waltham, Massachusetts, USA

Howden R, Andersen CR, Goldsbrough PB, Cobbett CS (1995b) A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana.* Plant Physiol **107:** 1067-1073

Howden R, Goldsbrough PB, Andersen CR, Cobbett CS (1995a) Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol **107:** 1059-1066

Huisman OC (1988) Seasonal colonization of roots of field-grown cotton by *Verticillium dahliae* and *V. tricorpus.* Phytopathology **78:** 708-716

Hutson RA, Mansfield JW (1980) A genetical approach to the analysis of mechanisms of pathogenicity in *Botrytis/Vicia* faba interactions. Physiol Plant Pathol **17:** 309-317

Hutson RA, Smith IM (1980) Phytoalexins and tyloses in tomato cultivars infected with *Fusarium oxysporum* f. sp. *lycopersici* or *Verticillium albo-atrum.* Physiol Plant Pathol **17:** 245-257

Hwu L, Huang CC, Chen DT, Lin A (2000) The action mode of the ribosome-inactivating protein α-sarcin. J. Biomed Sci **7:** 420-428

Ikawa M, Thomas Jr TV, Buckley LJ, Uebel JJ (1973) Sulfur and the toxicity of the red alga *Ceramium rubrum* to *Bacillus subtilis.* J Phycol **9:** 302-304

Imlay JA, Linn S (1988) DNA damage and oxygen radical toxicity. Science **240:** 1302-1309

Inouhe M, Ito R, Ito S, Sasada N, Tohoyama H, Joho M (2000) Azuki bean cells are hypersensitive to cadmium and do not synthesize phytochelatins. Plant Physiol **123:** 1029-1036

Isaac I (1967) Speciation in *Verticillium.* Annu Rev Phytopathol **5:** 201-222

Ito H, Hiraga S, Tsugawa H, Matsui H, Honma M, Otsuki Y, Murakami T, Ohashi Y (2000) Xylem-specific expression of wound-inducible rice peroxidase genes in transgenic plants. Plant Sci **155:** 85-100

Izac RR, Stierle DB, Sims JJ (1982) Sulfur and *N*-methylformamide from the marine red alga *Erythrophyllum delesserioides.* Phytochemistry **21:** 229

Jabs T, Tschöpe M, Colling C, Hahlbrock K, Scheel D (1997) Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in

triggering defense gene activation and phytoalexin synthesis in parsley. Proc Natl Acad Sci **94:** 4800-4805

Jach G, Görnhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Maas C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J **8:** 97-109

James DC, Rossiter JT (1991) Development and characteristics of myrosinase in *Brassica napus* during early seedling growth. Physiol Plant **82:** 163-170

Johnston DJ, Ramanathan V, Williamson B (1993) A protein from immature raspberry fruits which inhibits endopolygalacturonases from *Botrytis cinerea* and other micro-organsisms. J Exp Bot **44:** 971-976

Jolivet P (1993) Elemental sulfur in agriculture. *In* L De Kok, I Stulen, H Rennenberg, C Brunold, W Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, The Hague, The Netherlands, pp 193-206

Jolivet P, Bergeron E, Kien P (1992) Oxidation of elemental sulfur and sulfite by wheat chloroplasts. Phyton **32:** 59-62

Jolivet P, Bergeron E, Zimierski A, Meunier JC (1995) Metabolism of elemental sulphur and oxidation of sulphite by wheat and spinach chloroplasts. Phytochemistry **38:** 9-14

Jones HP (1934) Control of barley diseases. 1. Closed smut. Bull Ministry of Agriculture, Egypt, pp 1-19

Joyard J, Fores E, Blée E, Douce R (1988) Characterisation of elemental sulfur in isolated intact spinach chloroplasts. Plant Physiol **88:** 961-964

Juvik JA, Stevens MA, Rick CM (1982) Survey of the genus *Lycopersicon* for variability in α-tomatine content. HortScience **17:** 764-766

Kaminaka H, Morita S, Nakajima M, Masumura T, Tanaka K (1998) Gene cloning and expression of cytosolic glutathione reductase in rice (*Oryza sativa* L.) Plant Cell Physiol **39:** 1269-1280

Karapapa VK, Bainbridge BW, Heale JB (1997) Morphological and molecular characterization of *Vericillium longisporum* comb. nov., pathogenic to oilseed rape. Mycol Res **101:** 1281-1294

Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. Plant Cell **9:** 627-640

Kasbekar DP, Papavinasasundaram KG (1992) An inducible, non-degradative phytoalexin-resistance mechanism in *Dictyostelium discoideum* is supressed by mutations that alter membrane sterol composition. Appl Environ Microbiol **58:** 2071-2074

Katan J (1971) Symptomless carriers of the tomato *Fusarium* wilt pathogen. Phytopathology **61:** 1213-1217

Kato T, Kabuto C, Sasaki N, Tsunagawa M, Aizawa H, Fujita K, Kato Y, Kitahara Y (1973) Momilactones, growth inhibitors from rice, *Oryza sativa* L. Tetrahedron Lett **39:** 3861-3864

Kelly PJ, Bones A, Rossiter JT (1998) Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of *Brassica juncea*. Planta **206:** 370-377

Khatib A, El Aramouni A, Hassan A, Ravise A (1974) Accumulation de composés phénoliques et de phytoalexines par des variétés de tomate cultivées sur les sols infectés au Liban. Phytopathol Z **81:** 23-27

Kim H, Hirai MY, Hayashi H, Chino M, Naito S, Fujiwara T (1999) Role of *O*-acetyl-L-serine in the coordinated regulation of the expression of a soybean seed storage-protein gene by sulfur and nitrogen nutrition. Planta **209:** 282-289

Kim J, Leustek T (1996) Cloning and analysis of the gene for cystathionine γ-synthase from *Arabidopsis thaliana*. Plant Mol Biol **32:** 1117-1124

Kirk PM, Cannon PF, David JC (2001) Ainsworth and Bisby's Dictionary of the Fungi. CABI Bioscience, Egham, UK

Kistler HC, VanEtten HD (1984) Three non-allelic genes for pisatin demethylation in the fungus *Nectria haematococca*. J Gen Microbiol **130:** 2595-2603

Kjær A (1976) Glucosinolates in the Cruciferae. *In* J Vaughan, A MacLeod, B Jones, eds, The Biology and Chemistry of the Cruciferae. Academic Press, London, UK, pp 207-219

Klonus D, Hofgen R, Willmitzer L, Riesmeier JW (1994) Isolation and characterization of two cDNA clones encoding ATP-sulfurylases from potato by complementation of a yeast mutant. Plant J **6:** 105-112

Klughammer C, Hager C, Padan E, Schutz M, Schreiber U, Shahak Y, Hauska G (1995) Reduction of cytochromes with menaquinol and sulfide in membranes from green sulfur bacteria. Photosynthesis Res **43:** 27-34

Kocsy G, Galiba G, Brunold C (2001) Role of glutathione in adaption and signalling during chilling and cold acclimation in plants. Physiol Plant **113:** 158-164

Kodama O, Miyakawa J, Akatsuka T, Kiyosawa S (1992) Sakuranetin, a flavanone phytoalexin from ultraviolet-irradiated rice leaves. Phytochemistry **31:** 3807-3809

Kokubun T, Harborne JB (1994) A survey of phytoalexin induction in leaves of the Rosaceae by copper ions. Z. Naturforsch **49c:** 628-634

Koller W, Scheinpflug H (1987) Fungal resistance to sterol biosynthesis inhibitors: a new challenge. Plant Dis **71:** 1066-1074

Kombrink E, Somssich IE (1995) Defense responses of plants to pathogens. Adv Bot Res **21:** 2-34

Kömives T, Gullner G, Király Z (1998) Role of glutathione and glutathione-related enzymes in response of plants to environmental stress. *In* P Csermely, ed, Stress of Life. The New York Academy of Sciences, New York, USA, pp 251-258

Korolev N, Gindin G (1999) Vegetative compatibility in the entomopathogen *Verticillium lecanii*. Mycol Res **103:** 833-840

Koroleva OA, Davies A, Deeken R, Thorpe MR, Tomos D, Hedrich R (2000) Identification of a new glucosinolate-rich cell type in *Arabidopsis* flower stalk. Plant Physiol **124:** 599-608

Kouchi H, Hata S (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol Gen Genet **238:** 106-119

Kovari IA, Cobbett C, Goldsbrough PB (1997) Expression of tomato gamma-Glu-Cys synthetase in the *Arabidopsis cad2* mutant restores cadmium tolerance. Plant Physiol **114 (suppl):** 126

Kraus F, Schäfer W, Schmidt A (1984) Formation of elemental sulfur by *Chlorella fusca* during growth on L-cysteine ethylester. Plant Physiol **74:** 176-182

Kroon BAM, Scheffer RJ Elgersma (1991) Interactions between *Fusarium oxysporum* f. sp. *lycopersici* and callus of susceptible and resistant tomato lines: fungal growth and phytoalexin accumulation. J Phytopathol **132:** 57-64

Kuć J (1995) Phytoalexins, stress metabolism, and disease resistance in plants. Annu Rev Phytopathol **33:** 275-297

Kumar S (1998) Inheritance of resistance to *Fusarium* wilt (race 2) in chickpea. Plant Breeding **117:** 139-142

Kusai K, Yamanaka T (1973) The oxidation mechanisms of thiosulphate and sulphide in *Chlorobium thiosulphatophilum*: roles of cytochrome *c*-551 and cytochrome *c*-553. Biochim Biophys Acta **325:** 304-314

Kushnir S, Babiychuk E, Kampfenkel K, Belles-Boix E, Van Montagu M, Inzé D (1995) Characterization of *Arabidopsis thaliana* cDNAs that render yeasts tolerant toward the thiol-oxidizing drug diamide. Proc Natl Acad Sci **92:** 10580-10584

Kuzniak E, Sklodowska M (1999) The effect of *Botrytis cinerea* infection on ascorbate-glutathione cycle in tomato leaves. Plant Sci **148:** 69-76

Kylin H, Atuma S, Hovander L, Jensen S (1994) Elemental sulphur (S_8) in higher plants - biogenic or anthropogenic origin? Experientia **50:** 80-85

Lafitte C, Barthe JP, Montillet JL, Touzé A (1984) Glycoprotein inhibitors of *Colletotrichum lindemuthianum* endopolygalacturonase in near isogenic lines of *Phaseolus vulgaris* resistant and susceptible to anthracnose. Physiol Plant Pathol **25:** 39-53

Lam E, Kato N, Lawton M (2001) Programmed cell death, mitochondria and the plant hypersensitive response. Nature **411:** 848-853

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol **48:** 251-275

Lamoureux GL, Rusness DG (1993) Glutathione in the metabolism and detoxification of xenobiotics in plants. *In* L De Kok, I Stulen, H Rennenberg, C

Brunold, W Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, The Hague, The Netherlands, pp 221-237

Lancaster JE, Shaw ML (1994) Characterization of purified γ-glutamyl transpeptidase in onions: evidence for *in vivo* role as a peptidase. Phytochemistry **36:** 1351-1358

Lappartient AG, Touraine B (1996) Demand-driven control of root ATP sulfurylase activity and SO_4^{2-} uptake in intact canola. Plant Physiol **111:** 147-157

Lappartient AG, Vidmar JJ, Leustek T, Glass ADM, Touraine B (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. Plant J **18:** 89-95

Larsen RA (1988) The antioxidants of higher plants. Phytochemistry **27:** 969-978

Lauren DR, Watkinson JH (1985) Elemental sulphur analysis using high-performance liquid chromatography on 10-μm rigid polymer particles. J Chromatogr **348:** 317-320

Leah R, Tommerup H, Svendsen I, Mundy J (1991) Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J Biol Chem **266:** 1564-1573

Lebeda A, Luhová L, Sedlářová M, Jančová D (2001) The role of enzymes in plant-fungal pathogens interactions. Z Pflanzenk Pflanzen **108:** 89-111

Leckie D, Astley D, Crute IR, Ellis PR, Pink DAC, Boukema I, Monteiro AA, Dias S (1996) The location and exploitation of genes for pest and disease resistance in European gene bank collections of horticultural Brassicas. Acta Hort **407:** 95-102

Lee CW, Yoneyama K, Takeuchi Y, Konnai M, Tamogami S, Kodama O (1999) Momilactones A and B in rice straw harvested at different growth stages. Biosci Biotech Bioch **63:** 1318-1320

Leech MJ, May K, Hallard D, Verpoorte R, De Luca V, Christou P (1998) Expression of two consecutive genes of a secondary metabolic pathway in transgenic tobacco: molecular diversity influences levels of expression and product accumulation. Plant Mol Biol **38:** 765-774

Legendre L, Rueter S, Heinstein PF, Low PS (1993) Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine max*) cells. Plant Physiol **102:** 233-240

Legris-Delaporte S, Ferron F, Landry J, Coster C (1987) Metabolism of elemental sulfur in wheat leaves consecutive to its foliar application. Plant Physiol **85:** 1026-1030

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol **51:** 141-165

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:** 583-593

Li KN, Rouse DI and German TL (1994) PCR primers that allow intergeneric differentiation of ascomycetes and their application to *Verticillium* spp. Appl Environ Microbiol **60:** 4324-4331.

Li XZ, Starratt AN, Cuppels DA (1998) Identification of tomato leaf factors that activate toxin gene expression in *Pseudomonas syringae* pv. *tomato* DC3000. Phytopathology **88:** 1094-1100

Lin CM, Kim J, Du WX, Wei CI (2000) Bactericidal activity of isothiocyanates against pathogens on fresh produce. J Food Protect **63:** 25-30

Logan HM, Cathala N, Grignon C, Davidian JC (1996) Cloning of a cDNA encoded by a member of the *Arabidopsis thaliana* ATP sulfurylase multigene family. J Biol Chem **271:** 12227-12233

Louie PKK, Timpe RC, Hawthorne SB, Miller DJ (1993) Determination of elemental sulphur in coal by supercritical fluid extraction and gas chromatography with atomic emission detection. Fuel **72:** 225-231

Lu YP, Li ZS, Rea PA (1997) *AtMRP1* gene of *Arabidopsis* encodes a glutathione *S*-conjugate pump: isolation and functional definition of a plant ATP-binding cassette transporter gene. Proc Natl Acad Sci **94:** 8243-8248

Lucas JA (1998) Plant Pathology and Plant Pathogens. Blackwell Science, Oxford, UK

Lucas JA, Crute IR, Sherriff C, Gordon PL (1988) The identification of a gene for race-specific resistance to *Peronospora parasitica* (downy mildew) in *Brassica napus* var. *oleifera* (oilseed rape). Plant Pathol **37:** 538-545

Ludwig-Müller J, Bennett RN, Kiddle G, Ihmig S, Ruppel M, Hilgenberg W (1999) The host range of *Plasmodiophora brassicae* and its relationship to endogenous glucosinolate content. New Phytol **141:** 443-458

Lunn JE, Droux M, Martin J, Douce R (1990) Localization of ATP sulfurylase and *O*-acetylserine(thiol)lyase in spinach leaves. Plant Physiol **94:** 1345-1352

Lynch R, Kawchuk LM, Hachey J, Bains PS, Howard RJ (1997) Identification of a gene conferring high levels of resistance to *Verticillium* wilt in *Solanum chacoense*. Plant Dis **81:** 1011-1014

MacDonald WL, Hindal DF (1981) Life cycle and epidemiology of *Ceratocystis*. *In* M Mace, A Bell, C Beckman, eds, Fungal Wilt Diseases of Plants. Academic Press, London, UK, pp 113-144

Mace ME (1978) Contributions of tyloses and terpenoid aldehyde phytoalexins to *Verticillium* wilt resistance in cotton. Physiol Plant Pathol **12:** 1-11

Mace ME, Bell AA, Beckman CH (1976) Histochemistry and identification of disease-induced terpenoid aldehydes in *Verticillium*-wilt-resistant and –susceptible cottons. Can J Bot **54:** 2095-2099

Mace ME, Bell AA, Stipanovic RD (1978) Histochemistry and identification of flavanols in *Verticillium* wilt-resistant and –susceptible cottons. Physiol Plant Pathol **13:** 143-149

Mace ME, Stipanovich RD, Bell AA (1989) Histochemical localization of desoxyhemigossypol, a phytoalexin in *Verticillium dahliae*-infected cotton stems. New Phytol **111:** 229-232

Mach E (1879) Weinlaube, Berlin **2:** 113

Mackintosh SF, Matthews DE, VanEtten HD (1989) Two additional genes for pisatin demethylation and their relationship to the pathogenicity of *Nectria haematococca* on pea. Mol Plant-Microbe Interact **2:** 354-362

Madigan MT, Martinko JM, Parker J (1997) *Brock* Biology of Microorganisms. Prentice Hall, Upper Saddle River, New Jersey, USA

Maimann S, Wagner C, Kreft O, Zeh M, Willmitzer L, Höfgen R, Hesse H (2000) Transgenic potato plants reveal the indispensable role of cystathionine β-lyase in plant growth and development. Plant J **23:** 747-758

Maizel JV, Burkhardt HJ, Mitchell HK (1964) Avenacin, an antimicrobial substance isolated from *Avena sativa*. I. Isolation and antimicrobial activity. Biochemistry **3:** 424-426

Majeau N, Trudel J, Asselin A (1990) Diversity of cucumber chitinase isoforms and characterization of one seed basic chitinase with lysozyme activity. Plant Sci **68:** 9-16

Maloney AP, VanEtten HD (1994) A gene from the fungal plant pathogen *Nectria haematococca* that encodes the phytoalexin-detoxifying enzyme pisatin demethylase defines a new cytochrome P450 family. Mol Gen Genet **243:** 506-514

Mangini (1871) Weinlaube, Berlin **3:** 18

Manici LM, Lazzeri L, Palmieri S (1997) In-vitro fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. J Agr Food Chem **45:** 2768-2773

Manova E, Severac C, Andreev A, Clément R (1997) $NiPS_3$ intercalates as catalysts for the oxidation of sulfide ions: synthesis, catalytic activity and XPS study. J Catal **169:** 503-509

Mansfield J, Bennett M, Bestwick C, Woods-Tör A (1997) Phenotypic expression of gene-for-gene interactions involving fungal and bacterial pathogens: variation from recognition to response. *In* I Crute, E Holub, J Burdon, eds, The Gene-for-Gene Relationship in Plant-Parasite Interactions. CAB International, Wallingford, UK, pp 265-291

Mansfield JW (1982) The role of phytoalexins in disease resistance. *In* J Bailey, J Mansfield, eds, Phytoalexins. Blackie, Glasgow, UK, pp 253-282

Mansfield JW (2000) Antimicrobial compounds and resistance, the role of phytoalexins and phytoanticipins. *In* A Slusarenko, R Fraser, L Van Loon, eds,

Mechanisms of Resistance to Plant Diseases. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 325-370

Mansfield JW, Hargreaves JA, Boyle FC (1974) Phytoalexin production by live cells in broad bean leaves infected with *Botrytis cinerea*. Nature **251:** 316-317

Mansfield JW, Hutson RA (1980) Microscopical studies on fungal development and host responses in broad bean and tulip leaves inoculated with five species of *Botrytis*. Physiol Plant Pathol **17:** 131-144

Manzanares-Dauleux MJ, Divaret I, Baron F, Thomas G (2001) Assessment of biological and molecular variability between and within field isolates of *Plasmodiophora brassicae*. Plant Pathol **50:** 165-173

Mari M, Iori R, Leoni O, Marchi A (1993) *In vitro* activity of glucosinolate-derived isothiocyanates against postharvest fruit pathogens. Ann Appl Biol **123:** 155-164

Markosyan ZK (1970) Effect of pesticides on trees under hot-house conditions. Through Chem Abstr **73:** no. 130138u

Marlowe A (1982) Dry bubble of oyster mushroom caused by *Verticillium fungicola*. Plant Dis **66:** 859-860

Marmeisse R, Van den Ackerveken GFJM, Goosen T, De Wit PJGM, Van den Broek HWJ (1993) Disruption of the avirulence gene *avr9* in two races of the tomato pathogen *Cladosporium fulvum* causes virulence on tomato genotypes with the complementary resistance gene *Cf9*. Mol Plant-Microbe Interact **6:** 412-417

Marrs KA (1996) The functions and regulation of glutathione *S*-transferases in plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 127-158

Marsh RW (1929) Investigations on the fungicidal action of sulphur. III. Studies on the toxicity of sulphuretted hydrogen and on the interaction of sulphur with fungi. J Hort Sci **7:** 237-250

Martin H (1930) The hydrolysis of sulphur in relation to its fungicidal activity. J Agr Sci **20:** 32- 44

Martin H, Salmon ES (1932) The fungicidal properties of certain spray-fluids, IX. The fungicidal properties of the products of hydrolysis of sulphur. J. Agr Sci **22:** 595-616

Martin MN, Slovin JP (2000) Purified γ-glutamyl transpeptidases from tomato exhibit high affinity for glutathione *S*-conjugates. Plant Physiol **122:** 1417-1426

Martin-Hernandez AM, Dufresne M, Hugouvieux V, Melton R, Osbourn A (2000) Effects of targeted replacement of the tomatinase gene on the interaction of *Septoria lycopersici* with tomato plants. Mol Plant-Microbe Interact **13:** 1301-1311

Matthews DE, VanEtten HD (1983) Detoxification of the phytoalexin pisatin by a fungal cytochrome P-450. Arch Biochem Biophys **224:** 494-505

Mauch F, Dudler R (1993) Differential induction of distinct glutathione *S*-transferases of wheat by xenobiotcis and by pathogen attack. Plant Physiol **102:** 1193-1201

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol **88:** 936-942

Mauch F, Staehelin A (1989) Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell **1:** 447-457

May MJ, Leaver CJ (1994) *Arabidopsis thaliana* γ-glutamylcysteine synthetase is structurally unrelated to mammalian, yeast, and *Escherichia coli* homologs

May MJ, Vernoux T, Leaver C, Van Montagu M, Inzé D (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. J Exp Bot **49:** 649-667

Mayama S, Matsuura Y, Iida H, Tani T (1982) The role of avenalumen in the resistance of oat to crown rust, *Puccinia coronata* f. sp. *avenae*. Physiol Plant Pathol **20:** 189-199

Mayama S, Tani T (1982) Microspectrophotometric analysis of the location of avenalumin accumulation in oat leaves in response to fungal infection. Physiol Plant Pathol **21:** 141-149

Mayton HS, Olivier C, Vaughn SF, Loria R (1996) Correlation of fungicidal activity of *Brassica* species with allyl isothiocyanate production in macerated leaf tissue. Phytopathology **86:** 267-271

McCallan SEA, Wilcoxon F (1931) The fungicidal action of sulphur. II. The production of hydrogen sulphide by sulphured leaves and spores and its toxicity to spores. Contrib Boyce-Thompson Inst **3:** 13-38

McCance DJ, Drysdale RB (1975) Production of tomatine and rishitin in tomato plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Plant Pathol **7:** 221-230

McFadden HG, Chapple R, De Feyter R, Dennis E (2001) Expression of pathogenesis-related genes in cotton stems in response to infection by *Verticillium dahliae*. Physiol Mol Plant Pathol **58:** 119-131

McGarvey JA, Denny TP, Schell MA (1999) Spatial-temporal and quantitative analysis of growth and EPS 1 production by *Ralstonia solanacearum* in resistant and susceptible tomato cultivars. Phytopathology **89:** 1233-1239

McGrath MT (1996) Increased resistance to triadimefon and to benomyl in *Sphaerotheca fuliginea* populations following fungicide usage over one season. Plant Dis **80:** 633-639

McGrath SP, Johnston AE (1986) Sulphur – crop nutrient and fungicide. Span **29:** 57-59

McGuire MM, Hamers RJ (2000) Extraction and quantitative analysis of elemental sulfur from sulfide mineral surfaces by high-performance liquid chromatography. Environ Sci Technol **34:** 4651-4655

McLaughlin JR, Sherma J (1994) Quantitative HPTLC determination of elemental sulfur in sulfur topical medications. J Liq Chromatogr **17:** 4445-4450

McNabb HS (1974) Further speculation on the origin of the aggressive strain of *Ceratocystis ulmi*. Iowa Acad Sci **86:** 12

Mehlhorn H, Lelandais M, Korth HG, Foyer CH (1996) Ascorbate is the natural substrate for plant peroxidases. FEBS Lett **378:** 203-206

Meins F, Neuhaus JM, Sperisen C, Ryals J (1992) The primary structure of plant pathogenesis-related glucanohydrolases and their genes. *In* T Boller, F Meins, eds, Genes involved in Plant Defense. Springer-Verlag, Berlin, Germany, pp 245-282

Mengel K (1993) Consequences of sulfur deficiency. *In* L De Kok, I Stulen, H Rennenberg, C Brunold, W Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, The Hague, The Netherlands, pp 207-209

Mes JJ, Van Doorn AA, Wijbrandi J, Simons G, Cornelissen BJC, Haring MA (2000) Expression of the *Fusarium* resistance gene *I-2* colocalizes with the site of fungal containment. Plant J **23:** 183-193

Métraux JP, Boller T (1986) Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. Physiol Mol Plant Pathol **28:** 161-169

Meyer AC, Pataky JK, Juvik JA (1991) Partial resistance to northern leaf blight and Stewart's wilt in sweet corn germ plasm. Plant Dis **75:** 1094-1097

Meyer TE (1985) Isolation and characterization of soluble cytochromes, ferredoxins and other chromophoric proteins from the halophilic phototrophic bacterium *Ectothiorhodospira halophila*. Biochim Biophys Acta **806:** 175-183

Meyer TE, Bartsch RG, Cusanovich MA, Mathewson JH (1968) The cytochromes of *Chlorobium thiosulfatophilum*. Biochim Biophys Acta **153:** 143-151

Meyer TE, Kennel SJ, Tedro SM, Kamen MD (1973) Iron protein content of *Thiocapsa pfennigii*, a purple sulfur bacterium of atypical chlorophyll composition. Biochim Biophys Acta **292:** 634-643

Miao VP, Covert SF, VanEtten HD (1991b) A fungal gene for antibiotic resistance on a dispensible ("B") chromosome. Science **254:** 1773-1776

Miao VPW VanEtten HD (1992) Three genes for metabolism of the phytoalexin maakiain in the plant pathogen *Nectria haematococca*: meiotic instability and relationship to a new gene for pisatin demethylase. Appl Environ Microbiol **58:** 801-808

Miao VPW, Matthews DE, VanEtten HD (1991a) Identification and chromosomal locations of a family of cytochrome P-450 genes for pisatin detoxification in the fungus *Nectria haematococca*. Mol Gen Genet **226:** 214-223

Milford GFJ, Fieldsend JK, Porter AJR, Rawlinson CJ, Evans EJ, Bilsborrow P (1989) Changes in glucosinolate concentrations during the vegetative growth of single and double low cultivars of winter oil seed rape. Asp Appl Biol **23:** 83-90

Miller LP, McCallan SEA, Weed RM (1953) Quantitative studies on the role of hydrogen sulfide formation in the toxic action of sulfur to fungus spores. Contrib Boyce-Thompson Inst **17:** 151-171

Ming R, Brewbaker JL, Moon HG, Musket TA, Holley RN, Pataky JK, McMullen MD (1999) Identification of RFLP markers linked to a major gene, *sw1*, conferring resistance to Stewart's wilt in maize. Maydica **44:** 319-323

Mirelman D, Galun E, Sharon N, Lotan R (1975) Inhibition of fungal growth by wheat germ agglutinin. Nature **256:** 414-416

Mithen RF, Lewis BG, Fenwick GR (1986) In vitro activity of glucosinolates and their products against *Leptosphaeria maculans*. Trans Br Mycol Soc **87:** 433-440

Moesta P, Soyedl U, Lindner B, Grisebach HZ (1982) Detection of glyceollin at the cellular level in infected soybean by laser microprobe mass analysis. Zeit Naturforsch **37:** 748-751

Molina A, Ahl Goy P, Fraile A, Sánchez-Monge R, García-Olmedo F (1993) Inhibition of bacterial and fungal plant pathogens by thionins of types I and II. Plant Sci **92:** 169-177

Molina A, García-Olmedo F (1993) Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. Plant J **4:** 983-991

Moreno M, Segura A, García-Olmedo F (1994) Pseudothionin-St1, a potato peptide active against potato pathogens. Eur J Biochem **223:** 135-139

Morrissey JP, Osbourn AE (1999) Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiol Mol Biol Rev **63:** 708-724

Muckenschnabel I, Goodman BA, Williamson B, Lyon GD, Deighton N (2002) Infection of leaves of *Arabidopsis thaliana* by *Botrytis cinerea*: changes in ascorbic acid, free radicals and lipid peroxidation products. J Exp Bot **53:** 207-214

Mueller WC, Morgham AT (1996) Ultrastructure of the vascular responses of tobacco to *Fusarium oxysporum* f. sp. *nicotianae*. Can J Bot **74:** 1273-1278

Müller KO (1958) Studies on phytoalexins: I. The formation and the immunological significance of phytoalexin produced by *Phaseolus vulgaris* in response to infections with *Sclerotinia fructicola* and *Phytophthora infestans*. Aust J Biol Sci **11:** 275-300

Müller KO (1961) The phytoalexin concept and its methodological significance. Recent Adv Bot **1:** 396-400

Müller KO, Börger H (1940) Experimentelle Untersuchungen über die Phytophthora-Resistenz der Kartoffel. Arbeiten aus de Biologischen Reichsanstalt für Land- und Forstwirtschaft, Berin-Dahlem **23:** 189-231

Murillo M, Leustek T (1995) Adenosine-5'-triphosphate-sulfurylase from *Arabidopsis thaliana* and *Escherichia coli* are functionally equivalent but structurally and kinetically divergent: nucleotide sequence of two adenosine-5'-triphosphate-sulfurylase cDNAs from *Arabidopsis thaliana* and analysis of a recombinant enzyme. Arch Biochem Biophys **323:** 195-204

Nakamura T, Yamaguchi Y, Sano H (1999) Four rice genes encoding cysteine synthase: isolation and differential responses to sulfur, nitrogen and light. Gene **229:** 155-161

Nakayama M, Akashi T, Hase T (2000) Plant sulfite reductase: molecular structure, catalytic function and interaction with ferredoxin. J Inorg Biochem **82:** 27-32

Neuenschwander U, Suter M, Brunold C (1991) Regulation of sulfate assimilation by light and *O*-acetyl-L-serine in *Lemna minor* L. Plant Physiol **97:** 253-258

Newcombe G, Robb J (1988) The function and relative importance of the vascular coating response in highly resistant, moderately resistant and susceptible alfalfa infected *Verticillium albo-atrum*. Physiol Mol Plant Pathol **33:** 47-58

Newton AC, Caten CE (1988) Auxotrophic mutants of *Septoria nodorum* isolated by direct screening and by selection for resistance to chlorate. Trans Br Mycol Soc **90:** 199-207

Ng AYN, Blomstedt CK, Gianello R, Hamill JD, Neale AD, Gaff DF (1996) Isolation and characterization of a lowly expressed cDNA from the resurrection grass *Sporolobus stapfianus* with homology to eukaryote sulfate transporter proteins (accession no. X96761) (PGR96-032). Plant Physiol **111:** 651

Nicholson RL, Hammerschmidt R (1992) Phenolic compounds and their role in disease resistance. Annu Rev Phytopathol **30:** 369-389

Nicholson RL, Kollipara SS, Vincent JR, Lyons PC (1987) Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. Proc Natl Acad Sci **84:** 5520-5524

Niemann GJ, Baayen RP, Boon JJ (1990) Localization of phytoalexin accumulation and determination of changes in lignin and carbohydrate composition in carnation (*Dianthus caryophyllus* L.) xylem as a consequence of infection with *Fusarium oxysporum* f. sp. *dianthi*, by pyrolysis-mass spectrometry. Neth J Pl Path **96:** 133-153

Nieva Moreno MI, Isla MI, Sampietro AR, Vattuone MA (1997) Biological activity of the lectin from mature *Cyphomandra betacea* Sendt, Fruits. Eur J Cell Biol **74 (suppl 46):** 35

Noctor G, Arisi ACM, Jouanin L, Foyer CH (1998b) Manipulation of glutathione and amino acid biosynthesis in the chloroplast. Plant Physiol **118:** 471-482

Noctor G, Arisi ACM, Jouanin L, Foyer CH (1999) Photorespiratory glycine enhances glutathione accumulation in both the chloroplastic and cytosolic compartments. J Exp Bot **50:** 1157-1167

Noctor G, Arisi ACM, Jouanin L, Kunert KJ, Rennenberg H, Foyer CH (1998a) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot **49:** 623-647

Noctor G, Arisi ACM, Jouanin L, Valadier MH, Roux Y, Foyer CH (1997) The role of glycine in determining the rate of glutathione synthesis in poplar. Possible implications for glutathione production during stress. Physiol Plant **100:** 255-263

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol **49:** 249-279

Noctor G, Strolum M, Jouanin L, Kunert KJ, Foyer CH, Rennenberg H (1996) Synthesis of glutathione in leaves of transgenic poplar overexpressing γ-glutatmylcysteine synthetase. Plant Physiol **112:** 1071-1078

Noji M, Murakoshi I, Saito K (1994) Molecular cloning of a cysteine synthase cDNA from *Citrullus vulgaris* (watermelon) by genetic complementation in an *Escherichia coli* Cys^- auxotroph. Mol Gen Genet **244:** 57-66

Nuss L, Mahé A, Clark AJ, Grisvard J, Dron M, Cervone F, De Lorenzo G (1996) Differential accumulation of PGIP (polygalacturonase-inhibiting protein) mRNA in two near-isogenic lines of *Phaseolus vulgaris* L. upon infection with *Colletotrichum lindemuthianum*. Physiol Mol Plant Pathol **48:** 83-89

O'Connell RJ, Brown IR, Mansfield JW, Bailey JA, Mazau D, Rumeau D, Esquerré-Tugayé MT (1990) Immunocytochemical localization of hydroxyproline-rich glycoproteins accumulating in melon and bean at sites of resistance to bacteria and fungi. Mol Plant-Microbe Interact **3:** 33-40

Oeser B, Yoder OC (1994) Pathogenesis by *Cochliobolus heterostrophus* transformants expressing a cutinase-encoding gene from *Nectria haematococca*. Mol Plant-Microbe Interact **7:** 282-288

Osbourn A, Bowyer P, Lunness P, Clarke B, Daniels M (1995) Fungal pathogens of oat roots and tomato leaves employ closely related enzymes to detoxify different host plant saponins. Mol Plant-Microbe Interact **8:** 971-978

Osbourn AE (1996a) Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell **8:** 1821-1831

Osbourn AE (1996b) Saponins and plant defence – a soap story. Trends Plant Sci **1:** 4-9

Osbourn AE (1999) Antimicrobial phytoprotectants and fungal pathogens: a commentary. Fungal Genet Biol **26:** 163-168

Osbourn AE, Clarke BR, Lunness P, Scott PR, Daniels MJ (1994) An oat species lacking avenacin is susceptible to infection by *Gaeumannomyces graminis* var. *tritici*. Physiol Mol Plant Pathol **45:** 457-467

Owens RG (1960) Effects of elemental sulfur, dithiocarbamates, and related fungicides on organic acid metabolism of fungus spores. Develop Ind Microbiol **1:** 187-205

Pacholski ML, Cannon DM, Ewing AG, Winograd N (1999) Imaging of exposed headgroups and tailgroups of phospholipid membranes by mass spectrometry. J Am Chem Soc **121:** 4716-4717

Paiva NL, Sun Y, Dixon RA, VanEtten HD, Hrazdina G (1994) Molecular cloning of isoflavone reductase from pea (*Pisum sativum* L.): evidence for a 3*R*-isoflavone intermediate in (+)-pisatin biosynthesis. Arch Biochem Biophys **312:** 501-510

Papadopoulos YA, Christie BR, Boland GJ, Busch LV (1991) The use of histochemical analysis and stem colonization for distinguishing reactions of alfalfa to *Verticillium* wilt. Can J Bot **69:** 1275-1283

Papadopoulou K, Melton RE, Leggett M, Daniels MJ, Osbourn AE (1999) Compromised disease resistance in saponin-deficient plants. Proc Natl Acad Sci **96:** 12923-12928

Papavinasasundaram KG, Kasbekar DP (1993) Pisatin resistance in *Dictyostelium discoideum* and *Neurospora crassa*: comparison of mutant phenotypes. J Gen Microbiol **139:** 3035-3041

Papenbrock J, Schmidt A (2000) Characterization of a sulfurtransferase from *Arabidopsis thaliana*. Eur J Biochem **267:** 145-154

Parker GB, Hooker AL (1993) Inheritance of resistance to *Erwinia stewartii* in four inbred lines of dent corn: qualitative and quantitative analysis. Maydica **38:** 223-229

Passera C, Ghisi R (1982) ATP sulfurylase and *O*-acetylserine sulfydrylase in isolated mesophyll protoplasts and bundle sheath strands of S-deprived maize leaves. J Exp Bot **33:** 432-438

Patil RS, Ghormade V, Deshpande MV (2000) Chitinolytic enzymes: an exploration. Enzyme Microbe Tech **26:** 473-483

Pattaragulwanit K, Brune DC, Trüper HG, Dahl C (1998) Molecular genetic evidence for extracytoplasmic localization of sulfur globules in *Chromatium vinosum*. Arch Microbiol **169:** 434-444

Paul X, Rossignol Y (1982) Sulphur as a fungicide. Sulphur Agric **6:** 12-14

Pedras MSC, Khan AQ, Taylor JL (1997) Phytoalexins from Brassicas: overcoming plants' defenses. *In* P Hedin, R Hollingworth, E Masler, J Miyamoto, D Thompson, eds, Phytochemicals for Pest Control, ACS Symposium Series 658. American Chemical Society, Washington, USA, pp 155-166

Pegg GF (1974) *Verticillium* diseases. Rev Plant Pathol **53:** 157-182

Pegg GF (1981) Biochemistry and physiology of pathogenesis. *In* M Mace, A Bell, C Beckman, eds, Fungal Wilt Diseases of Plants. Academic Press, London, UK, pp 193-253

Pegg GF, Young DH (1981) Changes in glycosidase activity and their relationship to fungal colonization during infection of tomato by *Verticillium albo-atrum*. Physiol Plant Pathol **19:** 371-382

Pegg GF, Young DH (1982) Purification and characterization of chitinase enzymes from healthy and *Verticillium albo-atrum*-infected tomato plants, and from *V. albo-atrum*. Physiol Plant Pathol **21:** 389-409

Peng M, Kuć J (1992) Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf disks. Phytopathology **82:** 696-699

Perry NB, Foster LM (1994) Antiviral and antifungal flavonoids, plus a triterpene, from *Hebe cupressoides*. Planta Med **60:** 491-492

Petersen M, Van Der Straeten D, Bauw G (1995) Full-length cDNA clone from *Coleus blumei* (GenBank Z49150) with high similarity to cobalamine-independent methionine synthase. Plant Physiol **109:** 338

Peumans WJ, Van Damme EJM (1995) Lectins as plant defense proteins. Plant Physiol **109:** 347-352

Pezet R, Pont V (1977) Elemental sulfur: accumulation in different species of fungi. Science **196:** 428-429

Pierce MI, Cover EC, Richardson PE, Scholes VE, Essenberg M (1996) Adequacy of cellular phytoalexin concentrations in hypersensitively responding cotton leaves. Physiol Mol Plant Pathol **48:** 305-324

Pierce NB (1900) Peach leaf curl: its nature and treatment. US Department of Agriculture Division of Vegetable Physiology and Pathology Bull **20:** 1-194

Pilon-Smits EAH, Hwang S, Lytle CM, Zhu Y, Tai JC, Bravo RC, Chen Y, Leustek T, Terry N (1999) Overexpression of ATP sulfurylase in Indian mustard leads to increased selenate uptake, reduction, and tolerance. Plant Physiol **119:** 123-132

Porter AJR, Morton AM, Kiddle G, Doughty KJ, Wallsgrove RM (1991) Variation in the glucosinolate content of oilseed rape (*Brassica napus* L) leaves. 1. Effect of leaf age and position. Ann Appl Biol **118:** 461-467

Prange A, Arzberger I, Engemann C, Modrow H, Schumann O, Trüper HG, Steudel R, Dahl C, Hormes J (1999) In situ analysis of sulfur in the sulfur globules of phototrophic sulfur bacteria by X-ray absorption near edge spectroscopy. Biochim Biophys Acta **1428:** 446-454

Prasad TK, Anderson MD, Martin BA, Stewart CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell **6:** 65-74

Presley JT, Taylor EE (1969) Ontogeny of vessels influence the development of *Verticillium* in young cotton plants. Phytopathology **63:** 485-590

Prior A, Uhrig JF, Heins L, Weismann A, Lillig CH, Stoltze C, Soll J, Schwenn JD (1999) Structural and kinetic properties of adenylyl sulfate reductase from *Catharanthus roseus* cell cultures. Biochim Biophys Acta **1430:** 25-38

Prior P, Bart S, Leclercq S, Darrasse A, Anaïs G (1996) Resistance to bacterial wilt in tomato as discerned by spread of *Pseudomonas* (*Burholderia*) *solanacearum* in the stem tissues. Plant Pathol **45:** 720-726

Prusky D, Plumbley RA (1992) Quiescent infections of *Colletotrichum* in tropical and subtropical fruits. *In* J Bailey, M Jeger, eds, *Colletotrichum*: Biology, Pathology and Control. CAB International, Wallingford, UK, pp 289-307

Pu Z, Lu BY, Liu WY, Jin SW (1996) Characterization of the enzymatic mechanism of γ-momorcharin, a novel ribosome-inactivating protein with lower molecular weight of 11,500 purified from the seeds of bitter gourd (*Momordica charantia*). Biochem Bioph Res Co **229:** 287-294

Pueppke SG, VanEtten HD (1976) The relation between pisatin and the development of *Aphanomyces euteiches* in diseased *Pisum sativum*. Phytopathology **66:** 1174-1185

Puhalla JE, Bell AA (1981) Genetics and biochemistry of wilt pathogens. *In* M Mace, A Bell, C Beckman, eds, Fungal Wilt Diseases of Plants. Academic Press, London, UK, pp 146-192

Pullman GS, DeVay JE (1982a) Epidemiology of *Verticillium* wilt of cotton: a relationship between inoculum density and disease progression. Phytopathology **72:** 549-554

Pullman GS, DeVay JE (1982b) Epidemiology of *Verticillium* wilt of cotton: effects of disease development on plant phenology and lint yield. Phytopathology **72:** 554-559

Rahman MA, Abdullah H, Vanhaecke M (1999) Histopathology of susceptible and resistant *Capsicum annuum* cultivars infected with *Ralstonia solanacearum*. J Phytopathology **147:** 129-140

Ramsden JD (1995) Resistance of cocoa (*Theobroma cacao* L.) to wilt caused by *Verticillium dahliae* Kleb: studies on phytoalexin and pathogen distribution. Bsc (hons) Thesis. University of Bath, Bath, UK

Rask L, Andreasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol **42:** 93-113

Ratanapo S, Ngamjunyaporn W, Chulavatnatol M (2001) Interaction of a mulberry leaf lectin with a phytopathogenic bacterium, *P. syringae* pv. *mori*. Plant Sci **160:** 739-744

Rauser WE (1990) Phytochelatins. Annu Rev Biochem **59:** 61-86

Rauser WE (1995) Phytochelatins and related peptides. Structure, biosynthesis, and function. Plant Physiol **109:** 1141-1149

Ravanel S (1997) Methionine biosynthesis in higher plants: biochemical and molecular characterization of the transsulfuration pathway enzymes. C R Acad Sci Paris **320:** 497-504

Ravanel S, Droux M, Douce R (1995a) Methionine biosynthesis in higher plants. I. Purification and characterization of cystathionine γ-synthase from spinach chloroplasts. Arch Biochem Biophys **316:** 572-584

Ravanel S, Gakière B, Job D, Douce R (1998) Cystathionine γ-synthase from *Arabidopsis thaliana*: purification and biochemical characterization of the recombinant enzyme overexpressed in *Escherichia coli*. Biochem J **331:** 639-648

Ravanel S, Gakière B, Job D, Douce R (1998a) The specific features of methionine biosynthesis and metabolism in plants. Proc Natl Acad Sci **95:** 7805-7812

Ravanel S, Ruffet ML, Douce R (1995b) Cloning of an *Arabidopsis thaliana* cDNA encoding cystathionine β-lyase by functional complementation in *Escherichia coli*. Plant Mol Biol **29:** 875-882

Rawlins MR, Leaver CJ, May MJ (1995) Characterisation of an *Arabidopsis thaliana* cDNA encoding glutathione synthetase. FEBS Lett **376:** 81-86

Ray DT, Orum TV, Bigelow DM, Alcorn SM (1995) Selection of diploid and screening of polyploid guayule lines for *Verticillium* tolerance. Ind Crop Prod **4:** 303-310

Rea PA, Li ZS, Lu YP, Drozdowicz YM, Martinoia E (1998) From vacuolar GS-X pumps to multispecific ABC transporters. Annu Rev Plant Physiol Plant Mol Biol **49:** 727-760

Reichheld JP, Vernoux T, Lardon F, Van Montagu M, Inzé D (1999) Specific checkpoints regulate plant cell cycle progression in response to oxidative stress. Plant J **17:** 647-656

Reimmann C, VanEtten HD (1994) Cloning and characterization of the *PDA6-1* gene encoding a fungal cytochrome *P-450* which detoxifies the phytoalexin pisatin from garden pea. Gene **146:** 221-226

Reinartz M, Tschäpe J, Brüser T, Trüper HG, Dahl C (1998) Sulfide oxidation in the phototrophic sulfur bacterium *Chromatium vinosum*. Arch Microbiol **170:** 59-68

Rennenberg H, Grundel I (1985) Growth and hydrogen sulfide emission of photoheterotrophic tobacco cells supplied with L-cysteine as sole sulfur source. J Plant Physiol **120:** 47-56

Rennenberg H, Steinkamp R, Kesselmeier J (1981) 5-Oxo-prolinase in *Nicotinana tabacum*: catalytic properties and subcellular localization. Physiol Plant **62:** 211-216

Renosto F, Patel HC, Martin RL, Thomassian C, Zimmerman G, Segel IH (1993) ATP sulfurylase from higher plants: kinetic and structural characterization of the chloroplast and cytosol enzymes from spinach leaf. Arch Biochem Biophys **307:** 272-285

Resende MLV, Flood J, Cooper RM (1994) Host specialization of *Verticillium dahliae*, with emphasis on isolates from cocoa (*Theobroma cacao*). Plant Pathol **43:** 104-111

Resende MLV, Flood J, Cooper RM (1995) Effect of method of inoculation, inoculation density and seedling age at inoculation on the expression of resistance of cocoa (*Theobroma cacao* L.) to *Verticilium dahliae* Kleb. Plant Pathol **44:** 374-383

Resende MLV, Flood J, Ramsden JD, Rowan MG, Beale MH, Cooper RM (1996) Novel phytoalexins including elemental sulphur in the resistance of cocoa (*Theobroma cacao* L.) to Verticillium wilt (*Verticillium dahliae* Kleb.) Physiol Mol Plant Pathol **48:** 347-359

Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. Curr Opin Plant Biol **1:** 404-411

Rick CM, Uhlig JW, Jones AD (1994) High α-tomatine content in ripe fruit of Andean *Lycopersicon esculentum* var. *cerasiforme*: developmental and genetic aspects. Proc Natl Acad Sci **91:** 12877-12881

Ride JP (1983) Cell walls and other structural barriers in defence. *In* J Callow, ed, Biochemical Plant Pathology. John Wiley and Sons, Chichester, UK, pp 215-236

Robb J, Chen P, Stankiewicz A (2001) Tolerance to *Verticillium dahliae* in tomato. Proceedings of the 8th International *Verticillium* Symposium, Cordoba, Spain: pp 29

Robb J, Lee SW, Mohan R, Kolattukudy PE (1991) Chemical characterization of stress-induced vascular coating in tomato. Plant Physiol **97:** 528-536

Robb J, Powell DA, Street PFS (1987) Time course of wall-coating secretion in *Verticillium*- infected tomatoes. Physiol Mol Plant Pathol **31:** 217-226

Robb J, Powell DA, Street PFS (1989) Vascular coating: a barrier to colonization by the pathogen in *Verticillium* wilt of tomato. Can J Bot **67:** 600-607

Roberts MA, Wray JL (1996) Cloning and characterization of an *Arabidopsis thaliana* cDNA clone encoding an organellar isoform of serine acetyltransferase. Plant Mol Biol **30:** 1041-1049

Roberts TM, Fisher BEA (1985) Estimated trends in dry and wet sulphur deposition over the UK between 1970 and 1982 and effects on crop growth. J Sci Food Agric **36:** 262-263

Roberts WK, Selitrennikoff CP (1986) Isolation and partial characterization of two antifungal proteins from barley. Biochim Biophys Acta **880:** 161-170

Robertson J (1824) On the mildew and some diseases incident to fruit trees. Trans Hort Soc London **5:** 175

Robson G (1999) Hyphal cell biology. *In* R Oliver, M Schweizer, eds, Molecular Fungal Biology. Cambridge University Press, Cambridge, UK, pp 164-184

Roca MG (1997) Aspectos citológicos da variabilidade genética em *Glomerella cingulata* (Stonem.) Spauld and Schrenck f. sp. *phaseoli (Colletotrichum lindemuthianum* (Sacc. And Magn) Scribner). MSc Thesis. Universidade Federal de Lavras, Brazil, pp 82

Rodman JE (1991a) A taxonomic analysis of glucosinolate-producing plants, Part 1: Phenetics. Syst Bot **16:** 598-618

Rodman JE (1991b) A taxonomic analysis of glucosinolate-producing plants, Part 2: Cladistics. Syst Bot **16:** 619-629

Rogers EE, Glazebrook J, Ausubel FM (1996) Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis*-pathogen interactions. Mol Plant-Microbe Interact **9:** 748-757

Roine E, Wei W, Yuan J, Nurmiaho-Lassila EL, Kalkkinen N, Romantschuk M, He SY (1997) Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. Proc Natl Acad Sci **94:** 3459-3464

Rolland N, Droux M, Douce R (1992) Subcellular distribution of *O*-acetylserine(thiol)lyase in cauliflower (*Brassica oleracea* L.) inflorescence. Plant Physiol **98:** 927-935

Rolland N, Droux M, Lebrun M, Douce R (1993) *O*-acetylserine(thiol)lyase from spinach (*Spinacia oleracea* L.) leaf: cDNA cloning, characterization, and overexpression in *Escherichia coli* of the chloroplast isoform. Arch Biochem Biophys **300:** 213-222

Romeis T (2001) Protein kinases in the plant defence response. Curr Opin Plant Biol **4:** 407-414

Romeis T, Piedras P, Zhang S, Klessig DF, Hirt H, Jones JDG (1999) Rapid Avr9- and Cf-9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. Plant Cell **11:** 273-287

Römer S, d'Harlingue A, Camara B, Schantz R, Kuntz M (1992) Cysteine synthase from *Capsicum annuum* chromoplasts. J Biol Chem **267:** 17966-17970

Rommens CM, Kishore GM (2000) Exploiting the full potential of disease-resistance genes for agricultural use. Curr Opin Biotech **11:** 120-125

Rosa EAS, Rodrigues PMF (1999) Towards a more sustainable agriculture system: the effect of glucosinolates on the control of soil-borne diseases. J Hortic Sci Biotech **74:** 667-674

Rose AB, Last RL (1994) Molecular genetics of amino acid, nucleotide, and vitamin biosynthesis. *In* E Meyerowitz, C Somerville, eds, *Arabidopsis*. Cold Spring Harbor Lab Press, New York, USA, pp 835-879

Rossall S, Mansfield JW (1978) The activity of wyerone acid against *Botrytis*. Ann Appl Biol **89:** 359-362

Rossotti H (1998) Diverse Atoms, Profiles of the Chemical Elements. Oxford University Press, Oxford, UK

Rozan TF, Theberge SM, Luther G (2000) Quantifying elemental sulfur (S^0), bisulfide (HS^-) and polysulfides (S_x^{2-}) using a voltammetric method. Anal Chim Acta **415:** 175-184

Rüegsegger A, Brunold C (1993) Localization of γ-glutamylcysteine synthetase and glutathione synthetase activity in maize seedlings. Plant Physiol **101:** 561-566

Ruffet ML, Droux M, Douce R (1994) Purification and kinetic properties of serine acetyltransferase free of *O*-acetylserine(thiol)lyase from spinach chloroplasts. Plant Physiol **104:** 597-604

Russell AD, Hugo WB, Ayliffe GAJ (1992) Disinfection, Preservation and Sterilization. Blackwell Scientific Publications, London, UK, pp 114-133

Saito K, Kurosawa M, Tatsuguchi K, Takagi Y, Murakoshi I (1994b) Modulation of cysteine biosynthesis in chloroplasts of transgenic tobacco overexpressing cysteine synthase [*O*-acetylserine(thiol)lyase]. Plant Physiol **106:** 887-895

Saito K, Miura N, Yamazaki M, Tatsuguchi K, Kurosawa M, Kanda R, Noji M, Murakoshi I (1994a) Molecular cloning and expression of key enzymes for biosynthesis of cysteine and related secondary non-protein amino acids. Plant Cell Tiss Org **38:** 153-158

Saito K, Yokoyama H, Noji M, Murakoshi I (1995) Molecular cloning and characterization of a plant serine acetyltransferase playing a regulatory role in cysteine biosynthesis from watermelon. J Biol Chem **270:** 16321-16326

Salgado MO, Schwartz HF (1993) Physiological specialization and effects of inoculum concentration of *Fusarium oxysporum* f. sp. *phaseoli* on common beans. Plant Dis **77:** 492-496

Salgado MO, Schwartz HF, Brick MA (1993) Inheritance of resistance to a Colorado race of *Fusarium oxysporum* f. sp. *phaseoli* in common beans. Plant Dis **79:** 279-281

Sasahira T, Matsui K, Arai T (1993) An oxidation enzyme for hydrogen sulfide from *Hyphomicrobium neptunium* ATCC15444. Yakugaku Zasshi **113:** 79-83

Sbaghi M, Jeandet P, Bessis R, Leroux P (1996) Degradation of stilbene-type phytoalexins in relation to the pathogenicity of *Botrytis cinerea* to grapevines. Plant Pathol **45:** 139-144

Scaife A, Turner M, Wood P (1983) Diagnosis of mineral disorders in plants. Crown Publishing, New York, USA

Schäfer HJ, Haag-Kerwer A, Rausch T (1998) cDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy-metal accumulator *Brassica juncea* L.: evidence for Cd-induction of a putative mitochondrial γ-glutamylcysteine synthetase isoform. Plant Mol Biol **37:** 87-97

Schäfer W, Straney D, Ciuffetti L, VanEtten HD, Yoder OC (1989) One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science **246:** 247-249

Schaible L, Cannon OS, Waddoups V (1951) Inheritance of resistance to *Verticillium* wilt in a tomato cross. Phytopathology **41:** 986-990

Schlumbaum A, Mauch F, Vögeli U, Boiler T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature **324:** 365-367

Schmidt A (1982) A cysteine desulfhydrase from spinach leaves specific for D-cysteine. Z Pflanzenphysiol **107:** 301-312

Schmidt A (1987) D-cysteine desulfhydrase from spinach. Method Enzymol **143:** 449-453

Schmidt A, Jäger K (1992) Open questions about sulfur metabolism in plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 325-349

Schmidt TM, Arieli B, Cohen Y, Padan E, Strohl WR (1987) Sulfur metabolism in *Beggiatoa alba*. J Bacteriol **169:** 5466-5472

Schnathorst WC (1981) Life cycle and epidemiology of *Verticillium*. *In* M Mace, A Bell, C Beckman, eds, Fungal Wilt Diseases of Plants. Academic Press, London, UK, pp 81-111

Schnathorst WC, Mathre DE (1966) Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. Phytopathology **56:** 1155-1161

Schneider A, Rennenberg H (1992) Degradation of glutathione (GSH) in heterotrophic tobacco cells. Phyton **32:** 111-115

Schneider S, Bergmann L (1995) Regulation of glutathione synthesis in suspension cultures of parsley and tobacco. Bot Acta **108:** 34-40

Schnug E, Booth E, Haneklaus S, Walker KC (1995a) Sulphur supply and stress resistance in oilseed rape. Proc 9th Int Rapeseed Congress, Cambridge: 229-231

Schnug E, Haneklaus S (1998) Diagnosis of sulfur nutrition. *In* E Schnug, ed, Sulfur in Agroecosystems. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1-38

Schnug E, Haneklaus S, Borchers A, Polle A (1995b) Relations between sulphur supply and glutathione and ascorbate concentrations in *Brassica napus*. Z Pflanzenernähr Bodenk **158:** 67-69

Schröder P (1993) Plants as a source of atmospheric sulfur. *In* L De Kok, I Stulen, H Rennenberg, C Brunold, W Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, The Hague, The Netherlands, pp 253-270

Schütz B, De Kok LJ, Rennenberg H (1991) Thiol accumulation and cysteine desulfhydrase activity in H_2S-fumigated leaves and leaf homogenates of curcurbit plants. Plant Cell Physiol **32:** 733-736

Schütz M, Shahak Y, Padan E, Hauska G (1997) Sulfide-quinone reductase from *Rhodobacter capsulatus*. J Biol Chem **272:** 9890-9894

Sekiya J, Schmidt A, Wilson LG, Filner P (1982) Emission of hydrogen sulfide by leaf tissue in response to L-cysteine. Plant Physiol **70:** 430-436

Selitrennikoff CP (2001) Antifungal proteins. Appl Environ Microbiol **67:** 2883-2894

Sexton AC, Howlett BJ (1998) Characterization of a gene encoding cyanide hydratase in *Leptosphaeria maculans*, the causal agent of blackleg disease of oilseed Brassicas. Proceedings of 7th International Congress of Plant Pathology, Edinburgh, UK: pp 1.8.45

Sgherri CLM, Navari-Izzo F (1995) Sunflower seedlings subjected to increasing water deficit stress: oxidative stress and defence mechanisms. Physiol Plant **93:** 25-30

Shahak Y, Arieli B, Padan E, Hauska G (1992) Sulphide quinone reductase (SQR) activity in *Chlorobium*. FEBS Lett **299:** 127-130

Shahak Y, Klughammer C, Schreiber U, Padan E, Herrman I, Hauska G (1994) Sulfide-quinone and sulfide-cytochrome reduction in *Rhodobacter capsulatus*. Photosynthesis Res **39:** 175-181

Sharvelle EG (1961) The Nature and Uses of Modern Fungicides. Burgess, Minneapolis, Minnesota, USA

Shaw DV, Gubler WD, Hansen J (1997) Field resistance of California strawberries to *Verticillium dahliae* at three conidial inoculum concentrations. HortScience **32:** 711-713

Shaw DV, Gubler WD, Larson KD, Hansen J (1996) Genetic variation for field resistance to *Verticillium dahliae* evaluated using genotypes and segregating progenies of California strawberries. J Amer Soc Hort Sci **121:** 625-628

Shi J, Mueller WC, Beckman CH (1991) Ultrastructural responses of vessel contact cells in cotton plants resistant or susceptible to infection by *Fusarium oxysporum* f. sp. *vasinfectum*. Physiol Mol Plant Pathol **38:** 211-222

Shi J, Mueller WC, Beckman CH (1992) Vessel occlusion and secretory activities of vessel contact cells in resistant or susceptible cotton plants infected with *Fusarium oxysporum* f. sp. *vasinfectum*. Physiol Mol Plant Pathol **40:** 133-147

Skipp RA, Bailey JA (1976) The effect of phaseollin on the growth of *Colletotrichum lindemuthianum* in bioassays designed to measure fungitoxicity. Physiol Plant Pathol **9:** 253-263

Skipp RA, Selby C, Bailey JA (1977) Toxic effects of phaseollin on plant cells. Physiol Plant Pathol **10:** 221-227

Skipsey M, Andrews CJ, Townson JK, Jepson I, Edwards R (1999) Isolation of cDNA (AJ243813) and genomic clones (AJ243812) of glutathione synthetase containing plastidic targeting sequences from *Arabidopsis thaliana*. Plant Physiol **121:** 312

Slocum RD, Kaur-Sawhney R, Galston AW (1984) The physiology and biochemistry of polyamines in plants. Arch Biochem Biophys **235:** 283-303

Smart R St C, Skinner WM, Gerson AR (1999) XPS of sulphide mineral surfaces: metal-deficient, polysulphides, defects and elemental sulphur. Surf Interface Anal **28:** 101-105

Smirnoff N (1993) The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol **125:** 27-58

Smith CA, MacHardy WE (1982) The significance of tomatine in the host response of susceptible and resistant tomato isolines infected with two races of *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathology **72:** 415-419

Smith DA (1982) Toxicity of phytoalexins. *In* J Bailey, J Mansfield, eds, Phytoalexins. Blackie, Glasgow, UK, pp 218-252

Smith DA, Banks SW (1986) Biosynthesis, elicitation and biological activity of isoflavonoid phytoalexins. Phytochemistry **25:** 979-995

Smith DN, King WJ, Topper CP, Boma F, Cooper JF (1995b) Alternative techniques for the application of sulphur dust to cashew trees for the control of powdery mildew caused by the fungus *Oidium anacardii* in Tanzania. Crop Prot **14:** 555-560

Smith DR (1971) Inheritance of reaction to Stewart's disease (bacterial wilt) in dent corn. MSc Thesis. University of Illinois, Illinois, USA.

Smith FW, Ealing PM, Hawkesford MJ, Clarkson DT (1995a) Plant members of a family of sulfate transporters reveal functional subtypes. Proc Natl Acad Sci **92:** 9373-9377

Smith FW, Hawkesford MJ, Ealing PM, Clarkson DT, Vanden Berg PJ, Belcher AR, Warrilow AGS (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. Plant J **12:** 875-884

Smith IK (1985) Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. Plant Physiol **79:** 1044-1047

Smith IK, Kendall AC, Keys AJ, Turner JC, Lea PJ (1984) Increased levels of glutathione in a catalase-deficient mutant of barley (*Hordeum vulgare* L.). Plant Sci Lett **37:** 29-33

Smith IK, Vierheller TL, Thorne CA (1989) Properties and functions of glutathione reductase in plants. Physiol Plant **77:** 449-456

Snyder BA, Leite B, Hipskind J, Butler LG (1991) Accumulation of sorghum phytoalexins induced by *Colletotrichum graminicola* at the infection site. Physiol Mol Plant Pathol **39:** 463-470

Snyder BA, Nicholson RL (1990) Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. Science **248:** 1637-1638

Somssich IE, Hahlbrock K (1998) Pathogen defence in plants – a paradigm of biological complexity. Trends Plant Sci **3:** 86-90

Sörbo B (1957) A colorimetric method for the determination of thiosulfate. Biochim Biophys Acta **23:** 412-416

Soytong K, Kanokmedhakul S, Kukongviriyapa V, Isobe M (2001) Application of *Chaetomium* species (Ketomium ®) as a new broad spectrum biological fungicide for plant disease control: a review article. Fungal Divers **7:** 1-15

Spanu P (1997) HCF-1, a hydrophobin from the tomato pathogen *Cladosporium fulvum*. Gene **193:** 89-96

Stadtman ER (1990) Metal ion-catalyzed oxidation of proteins. Biochemical-mechanism and biological consequences. Free Radical Bio Med **9:** 315-325

Stakman EC (1915) Relation between *Puccinia graminis* and plants highly resistant to its attack. J Agric Res **4:** 193-299

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JDG (1995) Molecular genetics of plant disease resistance. Science **268:** 661-667

Stefess GC, Torremans RAM, De Schrijver R, Robertson LA, Kuenen JG (1996) Quantitative measurement of sulphur formation by steady-state and transient-state continuous cultures of autotrophic *Thiobacillus* species. Appl Microbiol Biotechnol **45:** 169-175

Steinberg G, Wedlich-Söldner R, Brill M, Schulz I (2000) Microtubules in the fungal pathogen *Ustilago maydis* are highly dynamic and determine cell polarity. J Cell Sci **114:** 609-622

Steinkamp R, Schweihofen B, Rennenberg H (1987) γ-Glutamyl-cyclotransferase in tobacco suspension cultures: catalytic properties and subcellular localization. Plant Physiol **69:** 499-503

Steinmetz MA, Trüper HG, Fischer U (1983) Cytochrome *c*-555 and iron-sulfur proteins of the non-thiosulfate-utilizing green sulfur bacterium *Chlorobium vibrioforme*. Arch Microbiol **135:** 186-190

Steudel R (1989) On the nature of the elemental sulfur produced by sulfur-oxidizing bacteria. *In* H Schlegel, B Bowien, eds, Biology of Autotrophic Bacteria. Science Technology Publications, Madison, Wisconsin, USA, pp 289-303

Steudel R (1996) Mechanism for the formation of elemental sulfur from aqueous silfide in chemical and microbiological desulfurization processes. Ind Eng Chem Res **35:** 1417-1423

Steudel R, Holdt G, Visscher PT, Van Gemerden H (1990) Search for polythionates in cultures of *Chromatium vinosum* after sulfide incubation. Arch Microbiol **153:** 432-437

Stevens RG, Creissen GP, Mullineaux PM (1997) Cloning and characterisation of a cytosolic glutathione reductase cDNA from pea (*Pisum sativum* L.) and its expression in response to stress. Plant Mol Biol **35:** 641-654

Stevens RG, Creissen GP, Mullineaux PM (2000) Characterisation of pea cytosolic glutathione reductase expressed in transgenic tobacco. Planta **211:** 537-545

Steventon LA, Okori P, Dixelius C (2001) An investigation of the susceptibility of *Arabidopsis thaliana* to isolates of two species of *Verticillium*. J Phytopathol **149:** 395-401

Stholasuta P, Bailey JA, Severin V, Deverall BJ (1971) Effect of bacterial inoculation of bean and pea leaves on the accumulation of phaseollin and pisatin. Physiol Plant Pathol **1:** 177-183

Stoessl A, Arditti J (1984) Orchid phytoalexins. *In* J Arditti, ed, Orchid Biology, Reviews and Perspectives, III. Cornell University Press, Ithaca, New York, USA, pp 151-175

Strohm M, Jouanin L, Kunert KJ, Pruvost C, Polle A, Foyer CH, Rennenberg H (1995) Regulation of glutathione synthesis in leaves of transgenic poplar (*Populus tremula* X *P. alba*) overexpressing glutathione synthetase. Plant J **7:** 141-145

Stromberg EL, Corden ME (1977) Fungitoxicity of xylem extracts from tomato plants resistant or susceptible to Fusarium wilt. Phytopathology **67:** 693-697

Suleman P, Tohamy AM, Saleh AA, Madkour MA, Straney DC (1996) Variation in sensitivity to tomatine and rishitin among isolates of *Fusarium oxysporum* f. sp. *lycopersici* and strains not pathogenic on tomato. Physiol Mol Plant Pathol **48:** 131-144

Suter M, Von Ballmoos P, Kopriva S, Den Camp RO, Schaller J, Kuhlemeier C, Schürmann P, Brunold C (2000) Adenosine 5'-phosphosulfate sulfotransferase and adenosine 5'-phosphosulfate reductase are identical enzymes. J Biol Chem **275:** 930-936

Takahashi H, Asanuma W, Saito K (1999) Cloning of an *Arabidopsis* cDNA encoding a chloroplast localizing sulphate transporter isoform. J Exp Bot **50:** 1713-1714

Takahashi H, Sasakura N, Noji M, Saito K (1996) Isolation and characterization of a cDNA encoding a sulphate transporter from *Arabidopsis thaliana*. FEBS Lett **392:** 95-99

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M, Hawkesford MJ, Saito K (2000) The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. Plant J **23:** 171-182

Takahashi H, Yamazaki M, Sasakura N, Watanabe A, Leustek T, De Almeida Engler J, Engler G, Van Montagu M, Saito K (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in *Arabidopsis thaliana*. Proc Natl Acad Sci **94:** 11102-11107

Talboys PW (1958) Association of tyloses and hyperplasia of the xylem with vascular invasion of the hop by *Verticillium albo-atrum*. Trans Brit Mycol Soc **41:** 249-260

Tang X, Rolfe SA, Scholes JD (1996) The effect of *Albugo candida* (white blister rust) on the photosynthetic and carbohydrate metabolism of leaves of *Arabidopsis thaliana*. Plant Cell Environ **19:** 967-975

Tausz M, Grill D (2000) The role of glutathione in stress adaption of plants. Phyton **40:** 111-118

Tegtmeier KJ, VanEtten HD (1982) The role of pisatin tolerance and degradation in the virulence of *Nectria haematococca* on peas: a genetic analysis. Phytopathology **72:** 608-612

Terras FRG, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Rees SB, Torrekens S, Van Leuven F, Vanderleyden J, Cammue BPA, Broekaert WF (1995) Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell **7:** 573-588

Tessier BJ, Mueller WC, Morgham AT (1990) Histopathology and ultrastructure of vascular responses in peas resistant or susceptible to *Fusarium oxysporum* f. sp. *pisi*. Phytopathology **80:** 756-764

Thangstad OP, Evjen K, Bones A (1991) Immunogold-EM localization of myrosinase in Brassicaceae. Protoplasma **161:** 84-93

Thangstad OP, Winge P, Husebye H, Bones A (1993) The myrosinase (thioglucoside glucohydrolase) gene family in Brassicaceae. Plant Mol Biol **23:** 511-524

Thatcher RW, Streeter LR (1925) The adherence to foliage of sulfur in fungicidal dusts and sprays. New York State Agricultural Experiment Station, Geneva, New York, Tech Bull **116:** 3-18

Thevissen K, Ghazi A, De Samblanx GW, Brownlee C, Osborn RW, Broekaert WF (1996) Fungal membrane responses induced by plant defensins and thionins. J Biol Chem **271:** 15018-15025

Thevissen K, Terras FRG, Broekaert WF (1999) Permealization of fungal membranes by plant defensins inhibits fungal growth. Appl Environ Microbiol **65:** 5451-5458

Thomma BPHJ, Nelissen I, Eggermont K, Broekaert WF (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Altemaria brassicicola*. Plant J **19:** 163-171

Thompson GA, Datko AH, AH, Mudd SH, Giovanelli J (1982) Methionine biosynthesis in *Lemna* – studies on the regulation of cystathionine gamma – synthase, *O*-phosphohomoserine sulfhydrylase, and *O*-acetylserine sulfhydrylase. Plant Physiol **69:** 1077-1083

Thoquet P, Olivier J, Sperisen C, Rogowsky P, Prior P, Anaïs G, Mangin B, Bazin B, Nazer R, Grimsley N (1996) Polygenic resistance of tomato plants to bacterial wilt in the French West Indies. Mol Plant-Microbe Interact **9:** 837-842

Tjamos EC, Smith IM (1974) The role of phytoalexins in the resistance of tomato to *Verticillium* wilt. Physiol Plant Pathol **4:** 249-259

Tjamos EC, Smith IM (1975) The expression of resistance *to Verticillium albo-atrum* in monogenically resistant tomato varieties. Physiol Mol Plant Pathol **6:** 215-225

Tokunaga J, Bartnicki-Garcia S (1971) Cyst wall formation and endogenous carbohydrate utilization during synchronous encystment of *Phytophthora palmivora* zoospores. Arch Microbiol **79:** 283-292

Toniazzo V, Mustin C, Portal JM, Humbert B, Benoit R, Erre R (1999) Elemental sulfur at the pyrite surfaces: speciation and quantification. Appl Surf Sci **143:** 229-237

Trojanowska MR, Osbourn AE, Daniels MJ, Threlfall DR (2001) Investigation of avenacin-deficient mutants of *Avena stringosa*. Phytochemistry **56:** 121-129

Tsror L, Nachmias A (1995) Significance of the root system in *Verticillium* wilt tolerance in potato and resistance in tomato. Isr J Plant Sci **43:** 315-323

Tsugita A (1971) Phage lysozyme and other lytic enzymes. *In* D Boyer, ed, The Enzymes, Vol 5. Academic Press, New York, USA, pp 344-411

Tsuji J, Jackson EP, Gage DA, Hammerschmidt R, Somerville SC (1992) Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv. *syringae*. Plant Physiol **98:** 1304-1309

Turcotte SB, Benner RE, Riley AM, Li J, Wadsworth ME, Bodily DM (1993) Surface analysis of electrochemically oxidized metal sulfides using Raman spectroscopy. J Electroanal Chem **347:** 195-205

Turner EM (1940) *Ophiobolus graminis* Sacc. var. *avenae*, var. N., as the cause of take-all or whiteheads of oats in Wales. Trans Brit Mycol Soc **24:** 269-281

Turner EM (1953) The nature of the resistance of oats to the take-all fungus. J Exp Bot **4:** 264-271

Turner EM (1961) An enzymic basis for pathogenic specificity in *Ophiobolus graminis*. J Exp Bot **12:** 169-175

Turner P, Barber C, Daniels M (1984) Behaviour of the transposons Tn5 and Tn7 in *Xanthomonas campestris* pv. *campestris*. Mol Gen Genet **195:** 101-107

Turner WL, Pallett KE, Lea PJ (1998) Cystathionine β-lyase from *Echinochloa colonum* tissue culture. Phytochemistry **47:** 189-196

Tweedy BG (1981) Inorganic sulfur as a fungicide. Residue Rev **78:** 43-68

Tweedy BG, Turner N (1966) The mechanism of sulfur reduction by conidia of *Monilinia fructicola*. Contrib Boyce-Thompson Inst **23:** 255-265

Ullmann P, Gondet L, Potier S, Bach TJ (1996) Cloning of *Arabidopsis thaliana* glutathione synthetase (*GSH2*) by functional complementation of a yeast *gsh2* mutant. Eur J Biochem **236:** 662-669

Uppal BN, Malelu JS (1928) A preliminary report on experiments in the control of grain smut of jowar (*Andropogon sorghum*). Agr J India **23:** 471-472

Van Damme EJM, Peumans WJ, Barre A, Rougé P (1998) Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit Rev Plant Sci **17:** 575-692

Van Leeuwen GCM, Van Kesteren HA (1998) Delineation of the three brown rot fungi of fruit crops (*Monilinia* spp.) on the basis of quantitative characteristics. Can J Bot **76:** 2042-2050

Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol **55:** 85-97

Van Parijs J, Broekaert WF, Goldstein IJ, Peumans WJ (1991) Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta **183:** 258-264

Van Parijs J, Joosen HM, Peumans WJ, Geuns JM, Van Laere AJ (1992) Effect of the *Urtica dioica* agglutinin on germination and cell wall formation of *Phycomyces blakesleeanus* Burgeff. Arch Microbiol **158:** 19-25

VanderMolen GE, Beckman CH, Rodehorst E (1987) The ultrastructure of tylose formation in resistant banana following inoculation with *Fusarium oxysporum* f. sp. *cubense*. Physiol Mol Plant Pathol **31:** 185-200

VanderMolen GE, Labavitch JM, DeVay JE (1986) *Fusarium*-induced vascular gels from banana roots – a partial chemical characterization. Physiol Plant **66:** 298-302

VanderMolen GE, Labavitch JM, Strand LL, DeVay JE (1983) Pathogen-induced vascular gels: ethylene as a host intermediate. Physiol Plant **59:** 573-580

VanEtten H, Temporini E, Wasmann C (2001) Phytoalexin (and phytoanticipin) tolerance as a virulence trait: why is it not required by all pathogens? Physiol Mol Plant Pathol **59:** 83-93

VanEtten HD, Funnell-Baerg D, Wasmann C, McCluskey K (1994b) Location of pathogenicity genes on dispensable chromosomes in *Nectria haematococca* MP VI. Anton Van Leeuwen **65:** 263-267

VanEtten HD, Mansfield JW, Bailey JA, Farmer EE (1994a) Two classes of plant antibiotics: phytoalexins versus "phytoanticipins". Plant Cell **6:** 1191-1192

VanEtten HD, Matthews DE, Matthews PS (1989) Phytoalexin detoxification: importance for pathogenicity and practical implications. Annu Rev Phytopathol **27:** 143-164

VanEtten HD, Matthews DE, Smith DA (1982) Metabolism of phytoalexins. *In* J Bailey, J Mansfield, eds, Phytoalexins. Blackie, Glasgow, UK, pp 181-217

VanEtten HD, Matthews PS (1984) Naturally occurring variation in the inducibility of pisatin demethylating activity in *Nectria haematococca* mating population VI. Physiol Plant Pathol **25:** 149-160

VanEtten HD, Matthews PS, Tegtmeier KJ, Dietert MF, Stein JI (1980) The association of pisatin tolerance and demethylation with virulence on pea *in Nectria haematococca*. Physiol Plant Pathol **16:** 257-268

VanEtten HD, Sandrock RW, Wasmann CC, Soby SD, McCluskey K, Wang P (1995) Detoxification of phytoanticipins and phytoalexins by phytopathogenic fungi. Can J Bot **73:** S518-S525

Vatamaniuk OK, Mari S, Lu YP, Rea PA (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis*: isolation and *in vitro* reconstitution. Proc Natl Acad Sci **96:** 7110-7115

Veena S, Padma DK (1995) A gravimetric procedure for determination of sulphur in disulphur dichloride. Indian J Chem **34A:** 414

Vernoux T, Wilson RC, Seeley KA, Reichheld JP, Muroy S, Brown S, Maughan SC, Cobbett SC, Van Montagu M, Inzé D, May MJ, Sung ZR (2000) The *ROOT MERISTEMLESS1/CADMIUM SENSITIVE2* gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell **12:** 97-109

Vidmar JJ, Schjoerring JK, Touraine B, Glass ADM (1999) Regulation of the *hvst1* gene encoding a high-affinity sulfate transporter from *Hordeum vulgare*. Plant Mol Biol **40:** 883-892

Vidmar JJ, Tagmount A, Cathala N, Touraine B, Davidian JCE (2000) Cloning and characterization of a root specific high-affinity sulfate transporter from *Arabidopsis thaliana*. FEBS Lett **475:** 65-69

Visser JM, Robertson LA, Van Verseveld HW, Gijs Kuenen J (1997) Sulfur production by obligately chemolithoautotrophic *Thiobacillus* species. Appl Environ Microbiol **63:** 2300-2305

Vögeli-Lange R, Wagner GJ (1996) Relationship between cadmium, glutathione and cadmium-binding peptides (phytochelatins) in leaves of intact tobacco seedlings. Plant Sci **114:** 11-18

Voisey CR, Slusarenko AJ (1989) Chitinase mRNA and enzyme activity in *Phaseolus vulgaris* (L.) increase more rapidly in response to avirulent than to virulent cells of *Pseudomonas syringae* pv. *phaseolicola*. Physiol Mol Plant Pathol **35:** 403-412

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kulper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res **23:** 4407-4414

Waldi D (1965) Spray reagents for thin-layer chromatography. *In* E Stahl, ed, Thin Layer Chromatography, a laboratory handbook. Springer-Verlag, London, UK pp 493

Wallsgrove RM, Lea PJ, Miflin BJ (1983) Intracellular localization of aspartate kinase and the enzymes of threonine and methionine biosynthesis in green leaves. Plant Physiol **71:** 780-784

Wang YH, Garvin DF, Kochian LV (2001) Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. Plant Physiol **127:** 345-359

Warkentin TD, Rashid KY, Xue AG (1996) Fungicidal control of powdery mildew in field pea. Can J Plant Sci **76:** 933-935

Wasmann CC, VanEtten HD (1996) Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of *Nectria haematococca* on pea. Mol Plant-Microbe Interact **9:** 793-803

Weckx JEJ, Clijsters HMM (1996) Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. Physiol Plant **96:** 506-512

Wefers H, Sies H (1983) Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen. Eur J Biochem **137:** 29-36

Wellhausen EJ (1937) Genetics of resistance to bacterial wilt in maize. Iowa Agricultural Experimental Station Res Bull **224:** 69-114

Weltring KM, Loser K, Weimer J (1998) Genetic instability of rishitin metabolism and tolerance and virulence on potato tubers of a strain of *Gibberella pulicaris*. J Phytopathol **146:** 393-398

Weltring KM, Schaub HP, Barz W (1995) Metabolism of pisatin stereoisomers by *Ascochyta rabiei* strains transformed with the pisatin demethylase gene of *Nectria haematococca* MP VI. Mol Plant-Microbe Interact **8:** 499-505.

Weltring KM, Turgeon BG, Yoder OC, VanEtten HD (1988) Isolation of a phytoalexin-detoxification gene from the plant pathogenic fungus *Nectria haematococca* by detecting its expression in *Aspergillus nidulans*. Gene **68:** 335-344

Wermter U, Fischer U (1983) Molecular properties of high potential iron sulfur protein of *Chromatium warmingii*. Z Naturforsch **38:** 968-971

Wilcoxon F, McCallan SEA (1930) The fungicidal action of sulfur. I. The alleged role of pentathionic acid. Phytopathology **20:** 391-417

Wilcoxon F, McCallan SEA (1931) The fungicidal action of sulphur. III. Physical factors affecting the efficiency of dusts. Contrib Boyce-Thompson Inst **3:** 509-528

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M, Inzé D, Van Camp W (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C_3 plants. EMBO J **16:** 4806-4816

Williams JS, Hall SA, Hawkesford MJ, Beale MH, Cooper RM (2002) Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen. Plant Physiol **128:** 150-159

Wingate VPM, Lawton MA, Lamb CJ (1988) Glutathione causes a massive and selective induction of plant defense genes. Plant Physiol **87:** 206-210

Winterbourn CC, Metodiewa D (1999) Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. Free Rad Biol Med **27:** 322-328

Wojtaszek P (1997) Oxidative burst: an early plant response to pathogen infection. Biochem J **322:** 681-692

Wu L, Joshi CP, Chiang VL (2000) A xylem-specific cellulose synthase gene from aspen (*Populus tremuloides*) is responsive to mechanical stress. Plant J **22:** 495-502

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell **10:** 1539-1550

Xu H, Heath MC (1998) Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus. Plant Cell **10:** 585-597

Xue J, Pihlgren U, Rask L (1993) Temporal, cell-specific, and tissue-preferential expression of myrosinase genes during embryo and seedling development in *Sinapis alba.* Planta **191:** 95-101

Yamaguchi Y, Nakamura T, Harada E, Koizumi N, Sano H (1997) Isolation and characterization of a cDNA encoding a sulfate transporter from *Araidopsis thaliana* (accession no. D89631) (PGR97-051). Plant Physiol **113:** 1463

Yang SF, Hoffman (1984) Ethylene biosynthesis and its regulation in higher plants. Ann Rev Plant Physiol **35:** 155-189

Yang Y, Shah J, Klessig DF (1997) Signal perception and transduction in plant defense responses. Genes Dev **11:** 1621-1639

Yano A, Suzuki K, Shinshi H (1999) A signaling pathway, independent of the oxidative burst, that leads to hypersensitive cell death in cultured tobacco cells includes a serine protease. Plant J **18:** 105-109

Yoshikawa M, Masago H (1982) Biochemical mechanism of glyceollin accumulation in soybean. *In* Y Asada, W Bushnell, S Ouchi, C Vance, eds, Plant Infection – The Physiological and Biochemical Basis. Japanese Society Press, Japan, pp 265-280

Yoshikawa M, Yamauchi K, Masago H (1978) Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. Physiol Plant Pathol **12:** 73-82

Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in *Arabidopsis* roots. Plant J **29:** 465-473

Youle D (1989) Pathogenicity factors and their induction in *Erwinia amylovora*. Ph D Thesis, University of Bath, Bath, UK

Young DH, Pegg GF (1981) Purification and characterization of 1,3-β-glucan hydrolases from healthy and *Verticillium albo-atrum*-infected tomato plants. Physiol Plant Pathol **19:** 391-417

Young HC (1922) The toxic property of sulphur. Ann Missouri Botan Garden **9:** 403-435

Youssefian S, Nakamura M, Sano H (1993) Tobacco plants transformed with the *O*-acetylserine (thiol) lyase gene of wheat are resistant to toxic levels of hydrogen sulphide gas. Plant J **4:** 759-769

Ypema HL, Ypema M, Gubler WD (1997) Sensitivity of *Uncinula necator* to benomyl, triadimefon, myclobutanil, and fenarimol in California. Plant Dis **81:** 293-297

Zenk MH (1996) Heavy metal detoxification in higher plants - a review. Gene **179:** 21-30

Zeyen RJ, Bushnell WR, Carver TLW, Robbins MP, Clark TA, Boyles DA, Vance CP (1995) Inhibiting phenylalanine ammonia lyase and cinnamylalcohol dehydrogenase suppresses *Mla1* (HR) but not *mlo5* (non-HR) barley powdery mildew resistances. Physiol Mol Plant Pathol **47:** 119-140

Zhang GP, Shi YL, Wang WP, Liu WY (1999) Cation channel formed at lipid bilayer by cinnamomin, a new type II ribosome-inactivating protein. Toxicon **37:** 1313-1322

Zhou N, Tootle TL, Glazebrook J (1999) *Arabidopsis PAD3*, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. Plant Cell **11:** 2419-2428

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J (1998) *PAD4* functions upstream from salicylic acid to control defense responses in *Arabidopsis*. Plant Cell **10:** 1021-1030

Appendix 1

Media

A1.1 Media for fungal growth

Czapek dox liquid medium and Czapek dox agar
(Oxoid Ltd, Basingstoke, UK)
Produce as instructed on the packet and autoclave at 121°C, 15 psi for 20 min.

Czapek dox liquid medium with 0.1% yeast
Prepare as Czapek dox liquid medium but add 10g yeast extract (Difco, Sparks, Maryland, USA) before autoclaving at 121°C, 15 psi for 20 min.

Czapek dox PLUS liquid medium and agar

Czapek dox liquid medium	33.4g
Yeast extract	2g
Malt extract (Oxoid)	2g
Mycological peptone (Oxoid)	2g
Casein hydrolysate (BDH, Lutterworth, UK)	2g

Add 14g of agar (Oxoid) if required and make up to 1L with distilled water. Heat to dissolve agar if agar is present and autoclave at 121°C, 15 psi for 20 min.

Czapek dox V8 complete supplement (CzV8CS) agar

Czapek dox agar	45.4g
$CaCO_3$ (BDH)	3g
Filtered V8 juice (Campbells soup company, Camden, New Jersey, USA) filtered through 2 layers of muslin	200 mL
Agar	10g

Make up to 1L with distilled water, heat to dissolve agar and autoclave at 121°C, 15 psi for 20 min After cooling to 50°C, add 50 mL filter sterile complete supplement stock solution.

<u>Complete supplement</u>

Casamino acids (Difco)	20g
Bacteriological peptone (Oxoid)	20g
Yeast extract	20g
Adenine hydrochloride (Sigma, Poole, UK)	3g
Biotin (Fluka, Gillingham, UK)	0.02g
Nicotinic acid (BDH)	0.02g
ρ-aminobenzoic acid (Sigma)	0.02g
Pyridoxine (Sigma)	0.02g
Thiamine (Sigma)	0.02g

Make up to 1L with distilled water and filter sterilise.

Czapek dox V8 complete supplement (CzV8CS) liquid medium

Czapek dox liquid medium	45.4g
Filtered V8 juice	200 mL

Adjust to pH 6 with 1M NaOH. Make up to 1L with distilled water and autoclave at 121°C, 15 psi for 20 min. After cooling to 50°C, add 50 mL of filter sterile complete supplement as for CzV8CS agar.

Mathur's liquid medium

Magnesium sulphate.$7H_20$ (BDH)	2.5g
Potassium dihydrogen orthophosphate (Fisher, Loughborough, UK)	2.7g
Yeast extract	1g
Bacteriological peptone	1g
Sucrose (BDH)	10g

Make up to 1L with distilled water and autoclave at 121°C, 15 psi for 20 min.

Pea broth

Boil 75g frozen peas in 250 mL distilled water for 20 min and filter through miracloth. Add:

Sucrose	10g
Asparagine (Fisher)	1g
Magnesium sulphate.$7H_20$	0.25g
Potassium dihydrogen orthophosphate	0.5g

Make up to 1L with distilled water and autoclave at 121°C, 15 psi for 20 min.

Potato dextrose agar (PDA)

(Oxoid)

Produce as instructed on the packet and autoclave at 121°C, 15 psi for 20 min.

Prune lactose yeast (PLY) liquid medium and prune lactose yeast agar (PLYA)

Prune extract	100 mL
Lactose (BDH)	5g
Yeast extract	1g

Add 20g of agar if required and make up to 1L with distilled water. Heat to dissolve agar if agar is present and autoclave at 121°C, 15 psi for 20 min.

Prune extract

Chop and stone 50 prunes, add 1L of distilled water, heat for 15 min in the microwave (full power), filter through muslin and autoclave at 121°C, 15 psi for 20 min.

V8 agar plus $CaCO_3$

Filtered V8 juice	167 mL
$CaCO_3$	2.5g
Agar	12.5g

Make up to 1L with distilled water, heat to dissolve agar and autoclave at 121°C, 15 psi for 20 min.

A1.2 Media for bacterial growth

Bacto-agar glucose triphenyl-tetrazolium chloride (BGT) agar

Bacteriological peptone	10g
Casamino acids	1g
Yeast extract	1g
Agar	14g

Make up to 1L with distilled water, heat to dissolve agar and autoclave at 121°C, 15 psi for 20 min.

After cooling to 50°C add the following filter sterile components:

Glucose (BDH) (20% stock solution)	25 mL
Triphenyl-tetrazolium chloride (TTC) (Sigma) (1% stock solution)	5 mL

Bacto-peptone (B) broth

Make up as for BGT agar but without agar, glucose or TTC.

Nutrient broth and Nutrient agar

(Oxoid)

Produce as instructed on the packet and autoclave at 121°C, 15 psi for 20 min.

Nutrient yeast glycerol broth (NYGB) and nutrient yeast glycerol agar (NYGA)

Bacteriological peptone	5g
Yeast Extract	3g
Glycerol (BDH)	20g

Add 14g of agar if required and make up to 1L with distilled water. Heat to dissolve agar if agar is present and autoclave at 121°C, 15 psi for 20 min.

Appendix 2
Molecular Biology Reagents and Solutions

A2.1 PCR amplification and electrophoresis of DNA

1M Tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 8)
Dissolve 121.1g of Tris base (Sigma, Poole, UK) in 800 mL of milli-Q water and adjust pH to 8 with concentrated HCl (Fisher, Loughborough, UK). Make up to 1L with milli-Q water and autoclave at 121°C, 15 psi for 20 min.

0.5M Ethylenediamine tetra-acetic acid disodium salt (EDTA) (pH 8)
Add 186.1g EDTA (Sigma) to 800 mL of milli-Q water and adjust pH to 8 with NaOH pellets (BDH, Lutterworth, UK). Make up to 1L with milli-Q water and autoclave at 121°C, 15 psi for 20 min. Note the EDTA will not dissolve completely until the pH reaches 8.

TE buffer

1M Tris buffer (pH 8)	10 mL
0.5M EDTA (pH 8)	2 mL

Make up to 1L with milli-Q water and autoclave at 121°C, 15 psi for 20 min.

1X TBE buffer

Tris base	10.8g
Boric acid (BDH)	5.5g
0.5M EDTA (pH 8)	4 mL

Make up to 1L with milli-Q water and autoclave at 121°C, 15 psi for 20 min.

Loading buffer

Orange G (Sigma)	0.025g
Bromophenol blue (Sigma)	0.025g
Xylene cyanol FF (Sigma)	0.025g
Ficoll 400 (Sigma)	1.5g

Make up to 10 mL with milli-Q water and filter sterilise.

A2.2 RNA Extraction, electrophoresis, Northern blotting, hybridisation and stripping

RNAse free water
In a fume hood add 100 μL of diethyl pyrocarbonate (DEPC) (Fluka, Gillingham, UK) to 1L of milli-Q water. Secure the container and shake vigorously. Loosen the lid and allow to stand overnight in the fume hood before autoclaving at 121°C, 15 psi for 20 min.

1M LiCl

LiCl (Sigma)	42.39g

Make up to 1L with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

10% Sodium dodecyl sulphate (SDS)

SDS (BDH)	100g

Make up to 1L with RNAse free water.

1M Tris buffer (pH 8)
As described in A2.1 but prepare with RNAse free water.

0.5M EDTA (pH 8)
As described in A2.1 but prepare with RNAse free water.

TLES buffer

1M Tris buffer (pH 8)	100 mL
1M LiCl	100 mL
0.5M EDTA (pH 8)	20 mL
10% SDS	100 mL

Make up to 1L with RNAse free water.

4M LiCl

LiCl	169.56g

Make up to 1L with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

70% ethanol

Absolute alcohol (Fisher)	700 mL

Make up to 1L with RNAse free water.

1X TBE buffer
As described in A2.1 but prepare with RNAse free water.

3M Sodium acetate (pH 5.2)
Add 408.1g of sodium acetate.$3H_20$ (Sigma) to 800 mL of milli-Q water and adjust pH to 5.2 with glacial acetic acid. Make up to 1L with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

10X MEN buffer

3-(*N*-morpholino)propanesulphonic acid (MOPS) (Sigma)	41.86g
Sodium acetate	4.1g
EDTA	5.84g

Dissolve in 800 mL of RNAse free water and adjust pH to 7 with concentrated HCl. Make up to 1L with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

1X MEN buffer

Prepare by dilution of 10X MEN solution with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

Loading buffer

As described in A2.1 but prepare with RNAse free water.

0.1M NaOH 1mM EDTA

NaOH	4g
0.5M EDTA (pH 8)	2 mL

Make up to 1L with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

10X SSC

NaCl	87.65g
Sodium citrate (BDH)	43.6g

Dissolve in 800 mL of RNAse free water and adjust pH to 7 with NaOH. Make up to 1L with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

Hybridisation buffer

di-Sodium hydrogen orthophosphate anhydrous (BDH)	48.2g
Sodium dihydrogen orthophosphate .$2H_20$ (BDH)	24.6g
SDS	70g
0.5M EDTA (pH 8)	20 mL

Make up to 1L with RNAse free water.

TE buffer

As described in A2.1 but prepare with RNAse free water.

5M NaCl

NaCl (BDH)	292.2g

Make up to 1L with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

STE buffer

0.5M EDTA (pH 8)	20 mL
1M Tris buffer (pH 8)	20 mL
5M NaCl	20 mL

Make up to 1L with RNAse free water and autoclave at 121°C, 15 psi for 20 min.

Sephadex G-50 STE

Add 1 tablespoon of Sephedex™ G-50 Fine (Amersham Pharmacia Biotech, Little Chalfont, UK) to 100 mL STE buffer and autoclave at 121°C, 15 psi for 20 min.

2X SSC 0.1% SDS wash solution

10X SSC	200 mL
10% SDS	10 mL

Make up to 1L with RNAse free water.

1X SSC 0.1% SDS wash solution

10X SSC	100 mL
10% SDS	10 mL

Make up to 1L with RNAse free water.

0.1% SDS

Prepare by dilution of 10% SDS solution with RNAse free water.

Appendix 3
Temporal Effects of *Verticillium dahliae* Infection on Sulphate and Thiol Levels in Tomato Plants (S. Hall, IACR Rothamsted, Harpenden, UK, unpublished data)

A3.1 Temporal effects of *V. dahliae* infection on sulphate levels in resistant tomato plants

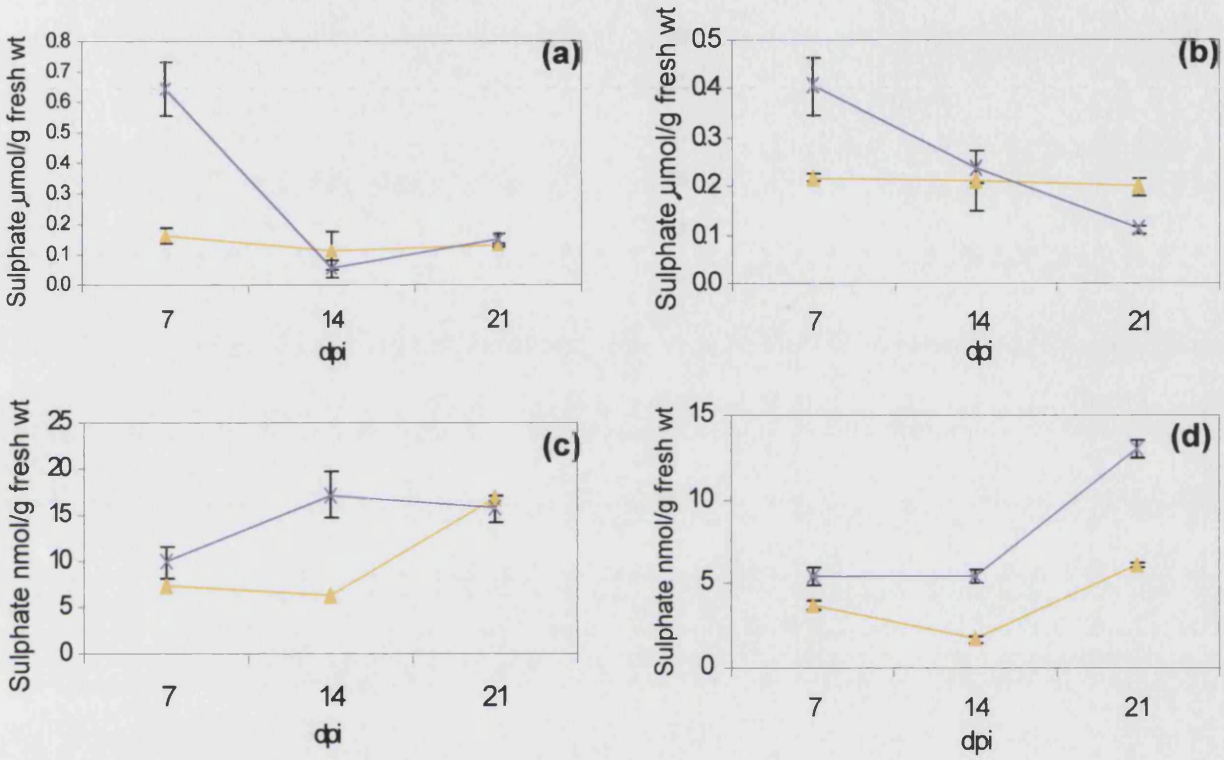

Figure A3.1 Temporal effects of *V. dahliae* infection on sulphate levels in resistant tomato plants. Values represent the mean of three replicates with SE. Plants were inoculated with either sterile water (▲) or *V. dahliae* (x). At 7, 14 and 21 dpi tissue samples were taken from root (a), total stem (up to node 8) (data not shown), stem vascular tissue (up to node 8) (b), leaf 4 (data not shown), leaf 8 (c) and leaf 15 (d) of plants and sulphate was estimated by HPLC.

A3.2 Temporal effects of *V. dahliae* infection on cysteine in xylem tissue of resistant and susceptible tomato plants

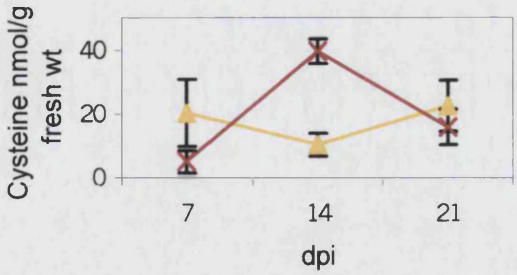

Figure A3.2 Temporal effects of *V. dahliae* infection on cysteine in xylem tissue of resistant and susceptible tomato plants. Values represent the mean of three replicates with SE. Plants were inoculated with either sterile water (▲) or *V. dahliae* (x). At 7, 14 and 21 dpi tissue samples were taken from root, total stem (up to node 8), stem vascular tissue (up to node 8), leaf 4, leaf 8 and leaf 15 of plants and cysteine was estimated by HPLC. Only stem vascular tissue (above) showed a significant change in cysteine levels on pathogen infection. Cysteine levels in pathogen-inoculated susceptible plants were not significantly different from those in control plants and are omitted for clarity. The data are representative of two comparable experiments.

A3.3 Temporal effect of *V. dahliae* infection on glutathione levels in resistant and susceptible tomato plants

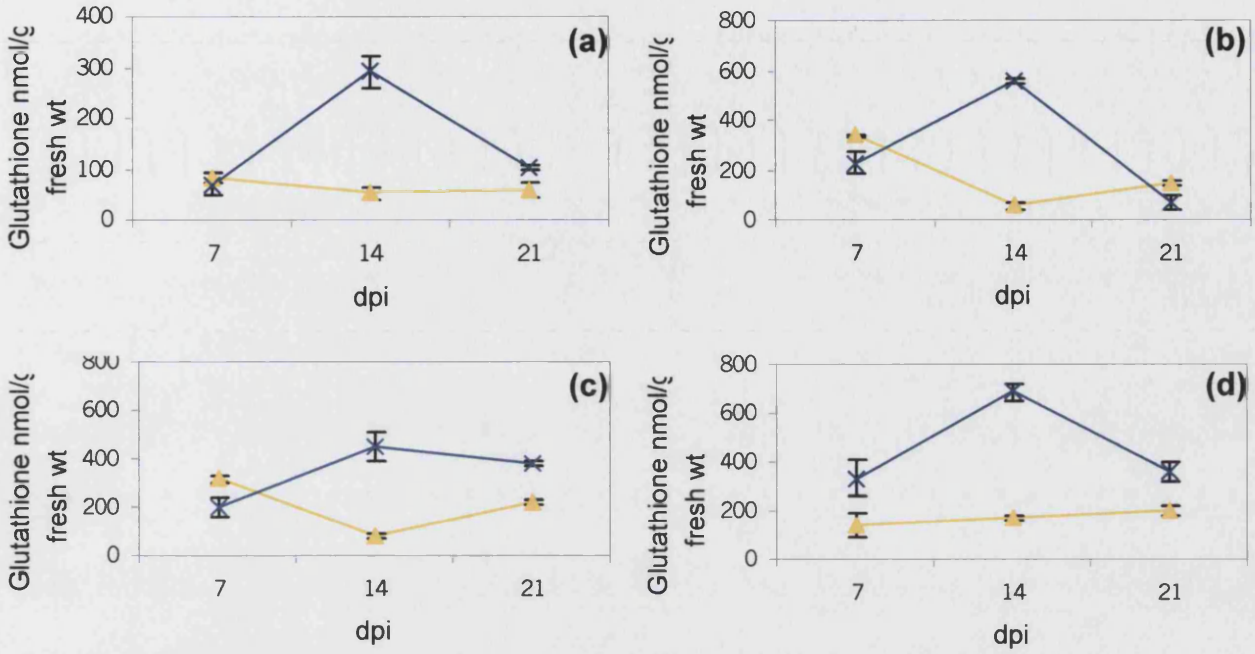

Figure A3.3 Temporal effect of *V. dahliae* infection on glutathione levels in resistant and susceptible tomato plants. Values represent the mean of three replicates with SE. Plants were inoculated with either sterile water (▲) or *V. dahliae* (x). At 7, 14 and 21 dpi tissue samples were taken from root (data not shown), total stem tissue (up to node 8) (data not shown) stem vascular tissue (up to node 8) (a), leaf 4 (b), leaf 8 (c) and leaf 15 (d) of plants and glutathione content was estimated by HPLC. Glutathione levels in pathogen-inoculated, susceptible plants were not significantly different from those in control plants and are omitted for clarity. A repeated experiment with stem vascular tissue produced similar data.

Thesis Submitted for a PhD at the University of Bath

"Sulphur is Really Interesting.... Honest" J. Williams

Introduction

Sulphur is mans oldest pesticide.... And we found it at BATH as part of the inate defense response in plants (or something).

Materials and Methods

1) Infect plants
2) Grind up
3) Extract
4) Go to long Ashton
5) Throw results away

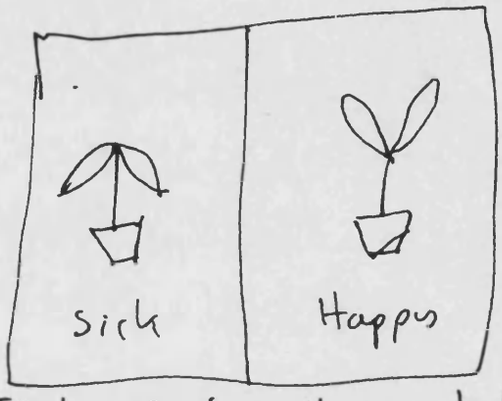

Fig 1. sick + Happy plants.

Results

Coming soon.... watch this space.

Conclusions.

It would of worked.... But for the following

1) Long Ashton
2) Long Ashton
3) Long Ashton
4) err...
5) Richard Cooper
6) Long Ashton
7) that's it!

Elemental Sulfur and Thiol Accumulation in Tomato and Defense against a Fungal Vascular Pathogen[1]

Jane S. Williams, Sharon A. Hall[2], Malcolm J. Hawkesford, Michael H. Beale, and Richard M. Cooper*

Department of Biology and Biochemistry, University of Bath, Bath, BA2 7AY United Kingdom (J.S.W., S.A.H., R.M.C.); Agriculture and Environment Division, IACR-Rothamsted, Hertfordshire, AL5 2JQ United Kingdom (M.J.H.); and Department of Agricultural Sciences, IACR, Long Ashton Research Station, University of Bristol, Long Ashton, Bristol, BS41 9AF United Kingdom (M.H.B.)

The occurrence of fungicidal, elemental S is well documented in certain specialized prokaryotes, but has rarely been detected in eukaryotes. Elemental S was first identified in this laboratory as a novel phytoalexin in the xylem of resistant genotypes of *Theobroma cacao*, after infection by the vascular, fungal pathogen *Verticillium dahliae*. In the current work, this phenomenon is demonstrated in a resistant line of tomato, *Lycopersicon esculentum*, in response to *V. dahliae*. A novel gas chromatography-mass spectroscopy method using isotope dilution analysis with ^{34}S internal standard was developed to identify unambiguously and quantify ^{32}S in samples of excised xylem. Accumulation of S in vascular tissue was more rapid and much greater in the disease-resistant than in the disease-susceptible line. Levels of S detected in the resistant variety (approximately 10 $\mu g\ g^{-1}$ fresh weight excised xylem) were fungitoxic to *V. dahliae* (spore germination was inhibited >90% at approximately 3 $\mu g\ mL^{-1}$). Scanning electron microscopy-energy dispersive x-ray microanalysis confirmed accumulation of S in vascular but not in pith cells and in greater amounts and frequency in the *Verticillium* spp.-resistant genotype. More intensive localizations of S were occasionally detected in xylem parenchyma cells, vessel walls, vascular gels, and tyloses, structures in potential contact with and linked with defense to *V. dahliae*. Transient increases in concentrations of sulfate, glutathione, and Cys of vascular tissues from resistant but not susceptible lines after infection may indicate a perturbation of S metabolism induced by elemental S formation; this is discussed in terms of possible S biogenesis.

Phytoalexins are defined as low-M_r, anti-microbial compounds that are both synthesized by and accumulate in plants after exposure to micro-organisms (Mansfield, 2000). A wide range of organic compounds such as phenolics and terpenoids has been identified as phytoalexins and they are synthesized from remote precursors. Although some phytoalexins are well known for their role in pathogen resistance in plants, the idea that elemental S (S^0), which has long been used by man as a protectant fungicide, may similarly function in defense, is relatively new (Cooper et al., 1996; Resende et al., 1996). In the typical, multiple phytoalexin response of resistant cultivars of *Theobroma cacao* to the vascular pathogen *Verticillium dahliae*, the most fungitoxic of four phytoalexins was S^0. It accumulated to fungitoxic levels in xylem and not in other tissues and persisted for >60 d. This was the first report of S^0 as an induced antimicrobial substance and of any inorganic element (other than structural functions in cell walls of calcium or silicon) contributing directly to active defense (Cooper et al., 1996). Elemental S formation is a property of many specialized prokaryotes (Schmidt et al., 1987; Visser et al., 1997; Reinartz et al., 1998) but until recently had only been described in eukaryotes for a few algae (Ikawa et al., 1973; Izak et al., 1982; Kraus et al., 1984). It appears however, that this phenomenon may be more widespread and elemental S could have a frequent role in pathogen resistance. S could function in preformed defenses as suggested by its occurrence in the cuticular wax of several gymnosperms and angiosperms (Kylin et al., 1994). It may also be associated with hypersensitivity, a rapid, localized apoptotic response, and the phenotypic expression of many major genes for disease resistance (Jabs and Slusarenko, 2000).

S afforded the unusual opportunity of cellular localization of an antimicrobial substance by coupled scanning electron microscopy-energy dispersive x-ray microanalysis (SEM-EDX). This revealed high concentrations of S in scattered xylem parenchyma (XP) cells, within vessel walls and in gels occluding vessels, areas in direct contact with the xylem-invading pathogen (Cooper et al., 1996). It is thought that the presence of elemental S in XP cells could reflect accumulation in hypersensitive cells, which lack metabolic capabilities. The death of scattered XP cells is typical for vascular diseases (Mace et al., 1976; Cooper, 1981), and other phytoalexins such as phaseollin and wyerone accumulate to high levels in ne-

[1] This work was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) studentship (to J.S.W.) and BBSRC grant no. 86/PO9332 (to S.A.H.). IACR receives grant-aided support from the BBSRC of the UK.

[2] Present address: Horticulture Research International, Plant Genetics and Biotechnology, Wellesbourne, Warwicks, CV35 9EF UK.

* Corresponding author; e-mail bssrmc@bath.ac.uk; fax 44–1225–826–779.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010687.

crotic cells after production by adjacent living cells (Mansfield, 2000). The persistence of S^0 in xylem tissues suggests unavailability to living cells, because wheat and spinach cells can metabolize elemental S in their chloroplasts (Legris-Delaporte et al., 1987; Joyard et al., 1988; Jolivet et al., 1995). This persistence also implies that localized accumulations were fungitoxic as many fungi can metabolize sublethal levels of S^0 (Beffa, 1993). Therefore, it is emerging that diverse plant species can produce and accumulate elemental S in structures that may play a key role in defense.

The major source of S for plants is sulfate, which is reduced in a multistep pathway, predominantly in the chloroplasts, to sulfide. It then combines to form Cys, some of which is subsequently converted to Met or glutathione; the latter is the major store and transportable form of non-protein reduced S (Schmidt and Jäger, 1992; Hell, 1997; Leustek and Saito, 1999; Hawkesford and Wray, 2000). Production of elemental S in eukaryotes is by an uncharacterized pathway, which may involve oxidation of sulfide. It has been postulated that a sulfide oxidase may be responsible for elemental S production in spinach chloroplasts (Joyard et al., 1988) and oxidation by cytochromes has been suggested in the green alga *Chlorella fusca* (Kraus et al., 1984). Both of these enzymes have been implicated in bacterial production of S^0 (Moriarty and Nicholas, 1970; Gray and Knaff, 1982; Cusanovich et al., 1991; Sasahira et al., 1992; Bang et al., 1995; Pattaragulwanit et al., 1998). The origin of the elemental S production in plants may be from glutathione or Cys degradation, possibly via the action of an, as yet uncharacterized, Cys desulfhydrase (Rennenberg et al., 1987; Schmidt, 1987). It is possible that sulfide is a by-product of the degradation of these thiols and it is this sulfide that is oxidized to form elemental S in a non-enzymic reaction (Steudel et al., 1986).

In the current work we demonstrate that elemental S is formed in tomato plants (*Lycopersicon esculentum*) in response to infection with *V. dahliae*. A comparison of a compatible and an incompatible interaction was made in isogenic lines lacking or containing the *Ve* gene for resistance to *Verticillium* spp. (Cooper and Wood, 1980; Diwan et al., 1999). This elemental S was extracted and quantified by gas chromatography-mass spectroscopy (GC-MS) as $^{32}S_8$, the most abundant isotope and common form of S^0. Tissue and cellular localization of S was similar to that in *T. cacao* (Cooper et al., 1996). S^0 accumulation in xylem of inoculated, disease-resistant tomatoes was coincident with or followed an increase in sulfate, Cys, and glutathione.

RESULTS

Colonization of Tomato Plants by *V. dahliae* and Resulting Disease Symptoms

Symptoms became apparent in infected GCR 26 (disease-susceptible) tomato plants at approximately 10 to 13 d postinoculation (dpi). Plants expressed symptoms of water stress (flaccidity of petioles and leaves, data not shown) around midday but recovered by evening through to early morning. Epinasty of lower petioles was also apparent at this time. In the next week, wilt symptoms became irreversible and severe. Flaccidity, chlorosis, and necrosis of the lower leaves progressed to successive leaves up the plant, adventitious roots were produced, and by 21 dpi plants were severely wilted and stunted. Resistant (GCR 218) plants had chlorotic areas on the lowest leaves, whereas other parts of the plant appeared healthy and they were a similar height to control plants. Removal of the stem epidermis of susceptible infected plants revealed brown discoloration of underlying vascular bundles in contrast to the cream-colored xylem tissues of healthy and resistant plants.

Rapid, acropetal hyphal colonization occurred in infected GCR 26 stems progressing from 5% of vessels infected at internode 1 at 13 dpi (when initial symptoms were evident) to 57% at 28 dpi (Fig. 1). Colonization of internode 8 was slow initially and none was evident at internode 15 up to 20 dpi, however invasion then progressed rapidly at both internodes to reach around 30% at 28 dpi. In GCR 218 plants, hyphal colonization by *V. dahliae* was sparse. Only approximately 0.3% of vessels contained hyphae in internode 1 at 13 dpi and hyphae were not detected in this or in higher internodes ≥20dpi. Control plants showed no colonization in any sections.

Percentage of vessels containing vascular occlusions (tyloses and gels) was also significantly higher (as determined by chi-square) in vascular tissues of *V. dahliae*-inoculated resistant plants than of suscep-

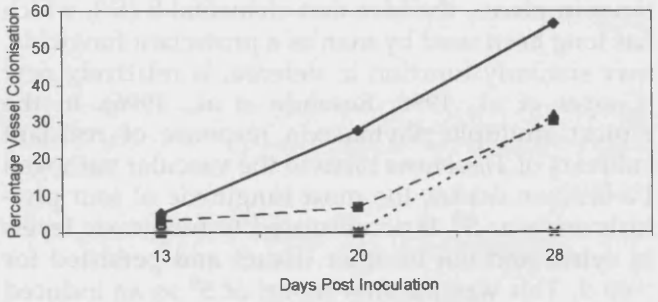

Figure 1. Colonization of susceptible (GCR 26) and resistant (GCR 218) tomato plants inoculated with *V. dahliae*. Analyses were performed at internodes 1 (♦), 8 (■), and 15 (▲) in susceptible plants and internode 1 (x) in resistant plants at 13, 20, and 28 dpi. Transverse sections of individual vascular bundles were cut from each internode of three replicate plants and percentage of vessels colonized was calculated. Points represent percentage of xylem vessels infected with fungal hyphae. Colonization for GCR 218 was only detectable at 0.3% in internode 1 at 13 dpi and so further data points for other internodes from the resistant variety are omitted for clarity. Chi-square tests at the 95% confidence level revealed significantly higher colonization of susceptible plants compared with resistant plants at internodes 1 and 8 at 13 and 20 dpi and in all internodes at 28 dpi.

tible plants until 28 dpi when a similar number of tyloses was present only at internode 1 of both treatments (data not shown). Control plants showed no vascular occlusion.

GC-MS Analysis of S_8 in Xylem from Susceptible and Resistant Tomato Lines

Xylem from control plants did not accumulate elemental S and none was detected in inoculated plants at 7 dpi (before stem colonization). Subsequently, inoculated susceptible plants showed a slow increase in S_8 reaching 1.88 ± 0.71 $\mu g\ g^{-1}$ at 21 dpi. In comparison, inoculated resistant plants showed a rapid and more substantial increase in elemental S and contained 10.4 ± 1.7 $\mu g\ g^{-1}$ at 21 dpi (Fig. 2).

SEM-EDX Localization of S in Vascular Tissue of Tomato Plants Inoculated with *V. dahliae*

Based on extent of colonization and the GC-MS analysis above, cryofixed and lyophilized, transverse and longitudinal sections were analyzed from the first internode of the tomato stems between 12 and 16 dpi and at 28 dpi to compare relative S levels and tissue and cellular distribution in resistant and susceptible, inoculated, and control plants. Cryofixed samples were coated in aluminum and lyophilized samples were coated in carbon before viewing by SEM. At 12 to 16 dpi general area analyses covering 25 vascular areas were made for each cryofixed treatment by x-ray analysis for the detection of S. S levels were recorded as "high" when the S peak was greater than 50% of the height of the potassium peak, which was always the predominant, endogenous element. Wherever a high level of S was detected, x-ray mapping was performed to enable visualization of any localized accumulations in the form of a dot map. These were compared with a secondary electron image to determine in which structure the S accumulation had occurred. Further localization studies were also attempted on lyophilized sections at 14 dpi and cryofixed and lyophilized samples at 28 dpi.

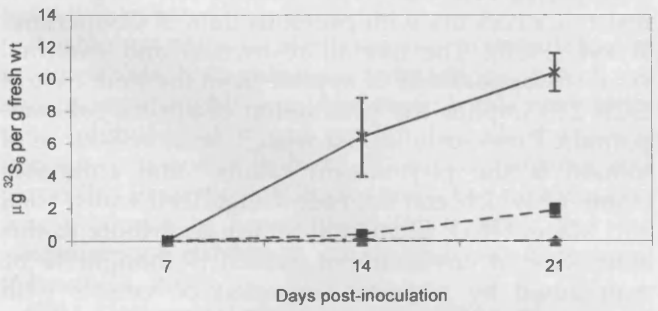

Figure 2. GC-MS analysis for S_8 of xylem tissue from resistant and susceptible tomato plants inoculated with *V. dahliae*. Xylem was harvested from three replicate control (♦) and inoculated (■) susceptible plants and control (▲) and inoculated (x) resistant plants at 7, 14, and 21 dpi for extraction and analysis by GC-MS. Values represent the mean with SE. Similar data were obtained in a repeated experiment.

At 12 to 16 dpi only very low levels of S were detected in all areas analyzed from control plants (Fig. 3a, i and ii). In inoculated, resistant plants 18 of 25 vascular areas examined showed high S (Fig. 3d, i and ii), but in the pith cells only very low levels, equivalent to that in control plants, were present (Fig. 3b, i and ii). In inoculated susceptible plants, S was low in the majority of vascular areas (17 of 25; Fig. 3c, i and ii).

For localization studies, cryofixed and lyophilized samples gave similar results. Because cryofixed samples could not be stored, lyophilized samples were used for subsequent analyses. In the vascular areas tested, from both resistant and susceptible stems at 12 to 16 dpi and at 28 dpi, those that had shown "low" S had no accumulations of S above background signal (Fig. 4a, i–iii) but in those that had shown "high" S, S had accumulated over much of the vascular tissue as evident from comparison with the background signal (Fig. 4b, i–iii). Occasionally, more intense localizations of S were detected in distinct XP cells (Fig. 4c, i and ii), gels (Fig. 4d, i and ii), tyloses, and vessel walls, in comparison with lower but still "high" levels detected in surrounding vascular structures.

Temporal Effect of Infection on Sulfate, Glutathione, and Cysteine Levels in Tomato Tissues

Roots, stems (both extracted vascular tissue and total stem samples), and leaves from nodes 4, 8, and 15 from control and inoculated resistant and susceptible plants were analyzed for sulfate, glutathione, and Cys levels by HPLC.

Sulfate levels were higher in pathogen-inoculated roots (Fig. 5a), stem vascular (Fig. 5b), and total stem tissue (data not shown) from the lower half of the plant than in corresponding control material at 7 dpi. This increase also occurred in leaves 4 and 8, but later at 14 dpi (Fig. 5c) and later still in leaf 15 at both 14 and 21 dpi (Fig. 5d).

In infected plants glutathione content of the stem vascular tissues and of leaves from the resistant but not susceptible line increased approximately 2- to 3-fold at 14 dpi (Fig. 6). No significant increase was detected in extracted entire stem or root samples (data not shown).

Cys levels followed a similar pattern and increased approximately 2- to 3-fold at 14 dpi but only in the vascular tissue of stems and only from the resistant genotype (Fig. 7).

Toxicity of Elemental S to *V. dahliae*

Elemental S was found to be highly toxic to *V. dahliae* spores and mycelium by the two assays used. The slide assay designed to investigate the inhibition

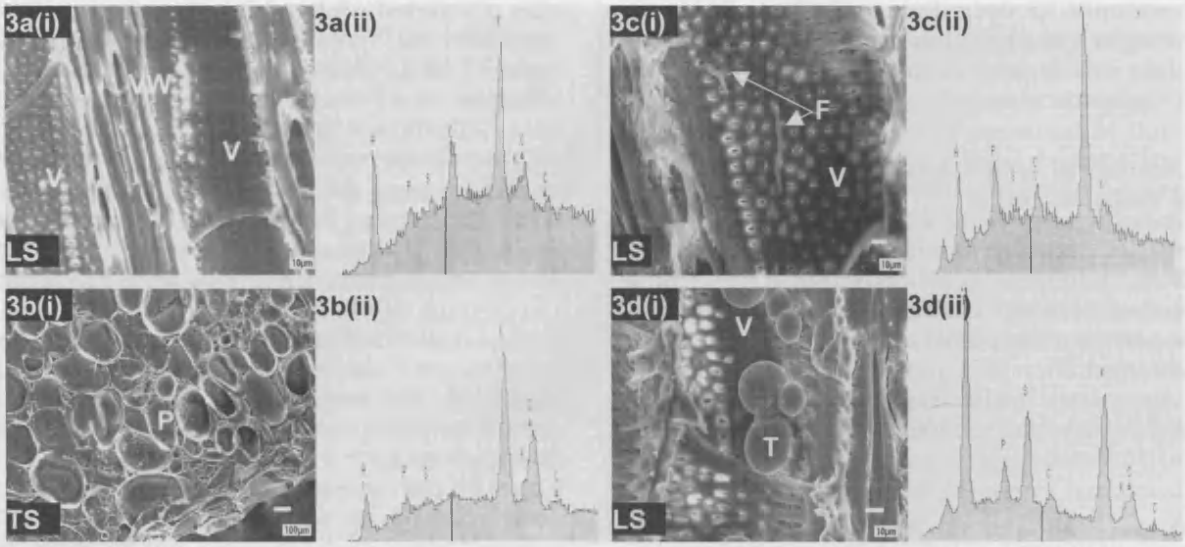

Figure 3. Relative S levels in resistant and susceptible *V. dahliae*-inoculated and control stems of tomato plants. Transverse and longitudinal sections from the base of the stem were analyzed at 12 to 16 dpi for the detection of "high" (>50% of K peak) or "low" (<50% of K peak) S. Twenty-five area x-ray analyses were made for each treatment and a representative scanning electron image (i) and spectrum from that image (ii) is shown (a–d). Very low levels of S were detected in all areas of control stems analyzed (a, i and ii) and in the central pith cells from resistant inoculated plants (b, i and ii). In susceptible, inoculated plants most vascular areas contained low S (c, i and ii) in comparison to resistant, inoculated plants where the majority of vascular areas showed high S (d, i and ii). There were more vascular occlusions (gels and tyloses [d, i]) evident in the resistant vascular tissues at this time than in the susceptible line in which many vessels contained fungal hyphae (c, i). Note the aluminum peak derives from the coating evaporated onto the sample. V, Vessel lumen; VW, vessel wall; F, fungal hypha; T, tylose; P, stem pith cell; XP, xylem parenchyma cell; VG, vascular gel; TS, transverse section; and LS, longitudinal section.

of spore germination by S^0 revealed >90% inhibition of spore germination at ≥3.125 μg mL^{-1} (Fig. 8). The thin layer chromatography (TLC) bioassay was designed to investigate toxicity of elemental S to spore germination initially and then to mycelial growth resulting from germination of spores in areas surrounding the zones of S application. Some inhibition of spore germination was evident at 4 d at concentrations of 3.125 and 6.25 μg mL^{-1} as fungal growth was less dense than in surrounding areas and control spots. Between 12.5 and 50 μg mL^{-1}, there was clear inhibition. From 100 μg mL^{-1} to 8,000 μg mL^{-1} inhibition also extended beyond the area of application suggesting that S may also act at a distance (Fig. 9). The patterns of inhibition did not change even after 40 d suggesting that mycelial growth (from surrounding germinated spores) into S treated zones was also inhibited at 3.125 μg mL^{-1} S and above.

DISCUSSION

To date there are few examples of elemental S production by eukaryotes. The discovery of S in resistant lines of tomato and previously of *T. cacao*, in sufficient quantities, in the right place, and at the right time potentially to inhibit *V. dahliae* are the first to implicate the element in induced disease resistance (Cooper et al., 1996; Resende et al., 1996). Clearly, tomato offers a more tractable model for genetical and biochemical dissection.

Related plant families generally make use of chemically related compounds for defense. S is perhaps the only known phytoalexin that is produced by different taxa, but this may reflect that it is the only known inorganic antimicrobial agent produced by plants (Dixon, 2001).

Inoculation of near isogenic disease-resistant and -susceptible tomato lines resulted in rapid, acropetal, systemic spread of *V. dahliae* in susceptible GCR 26, whereas colonization of the resistant variety (GCR 218) was restricted to basal internodes and was very sparse (approximately 0.3% vessels infected at the first internode). This expression of the *Ve* gene for resistance concurs with previous data of Cooper and Wood (1980). The overall restriction and eventual visual disappearance of hyphae from the stem base of GCR 218 implies the production of antifungal compounds. Previously linked with disease resistance of tomato is the phytoalexin rishitin and chitinases (some of which can degrade fungal cell walls) (Bell and Mace, 1981). Elemental S may contribute to this antimicrobial environment, which is thought to be maintained by physical occlusion of vessels with tyloses and gels (Cooper, 2000). In this study, tyloses were abundant in the incompatible interaction but significantly less in the infected xylem of the susceptible genotype.

Kinetics and levels of S accumulation were revealed accurately for the first time as a result of the development of a method for accurate quantification

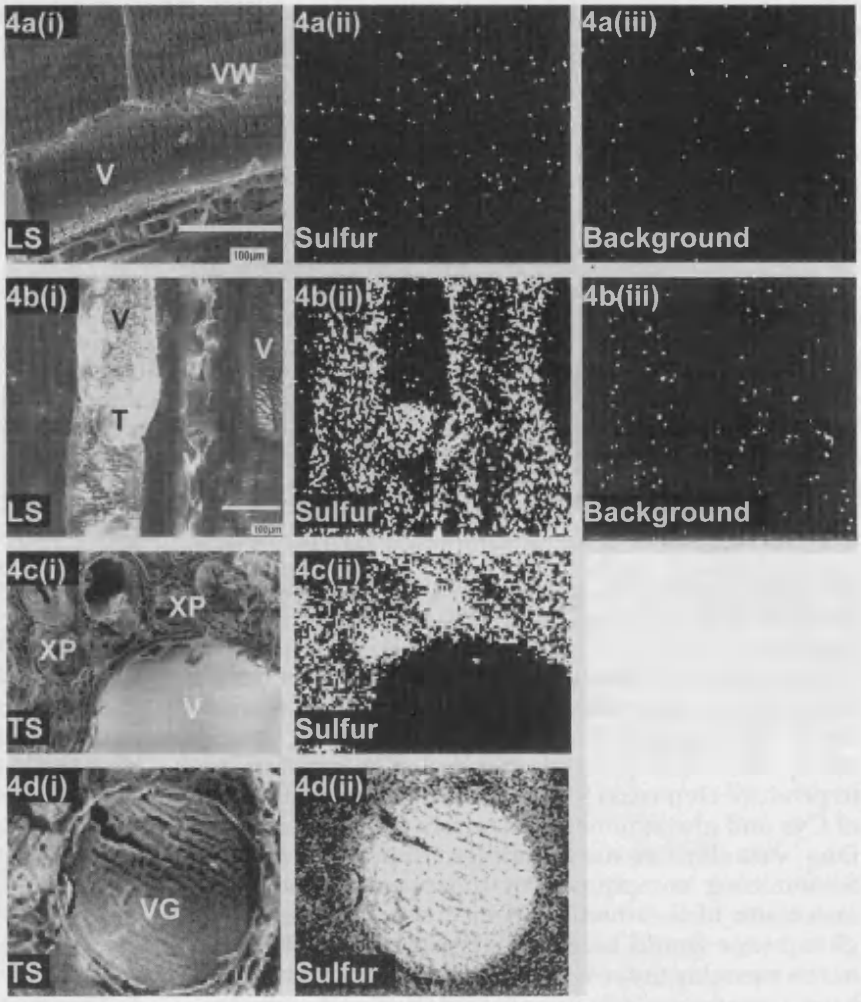

Figure 4. Distribution of S in vascular tissues of *V. dahliae*-inoculated and control stems of tomato plants. Transverse and longitudinal sections from the base of the stem were analyzed at 12 to 16 and 28 dpi for the detection of "high" (> 50% of K peak) or "low" (<50% of K peak) S. Wherever "high" S was detected an x-ray dot map was produced for localization of S. Representative SEM images are shown (i) with corresponding dot maps for S (ii) and background noise from the analyzer (iii; a–d). No accumulations of S were found in control samples above background (a, i–iii). In most samples that had high S, S was present over most of the vascular tissue in comparison to background signal (b, i–iii). Note accumulation of S in the tylose (b, ii). Occasionally, there were more intense spots of S in certain structures such as XP cells (c, i and ii) and gels (d, i and ii). For abbreviations see Figure 3 legend.

of $^{32}S_8$. S accumulation resembled that of various organic phytoalexins, with a more rapid and intensive production in the resistant than in the susceptible host; a pattern also suggested by SEM-EDX. This differential response is especially remarkable as it was inversely related to the amount of pathogen; fungal biomass in resistant xylem was negligible in contrast to the extensive colonization of xylem in the susceptible line.

Analogous patterns of phytoalexin accumulation in incompatible host-pathogen interactions, which result in rapid highly localized toxic levels coincident with inhibition of fungal growth, include the isoflavanoids phaseollin in bean (*Phaseolus vulgaris*) and glyceollin in soybean (*Glycine max*), the furanoacetylene wyerone in broad bean (*Vicia faba*), and the sesquiterpene rishitin in potato (*Solanum tuberosum*) (Mansfield, 2000).

SEM-EDX revealed that accumulation of S as the element and/or as organically bound S was widespread in tomato vascular tissue undergoing an incompatible interaction with *V. dahliae*. Occasional localizations were detected in scattered XP cells, vascular gels, and in xylem vessel walls in pathogen-inoculated plants and were detected in both cryo-fixed and lyophilized sections. These zones corresponded closely with the results of SEM-EDX on vascular tissue from resistant *T. cacao* plants inoculated with *V. dahliae* (Cooper et al., 1996). Terpenoid aldehyde phytoalexins of cotton are similarly formed in XP cells and these along with phytoalexins of some other species are exuded into xylem vessels to impregnate vascular occlusions (Bell and Mace, 1981). Impregnation of these structures with elemental S would be of direct relevance to resistance against vascular fungi by providing an effective barrier to vertical and lateral spread, which characterizes their mode of invasion (Cooper, 2000). Even based on the amounts of S^0 detected in entire tissue extracts (probably containing only a small proportion of S-containing XP cells), which after 14 and 21 dpi were approximately 6 and 10 $\mu g\ g^{-1}$, respectively, levels were greater than that required for inhibition of *V. dahliae* spore germination and hyphal growth; both structural forms are produced in infected xylem. Calculations of amounts of other phytoalexins such as phaseollin, have revealed that whole tissue extracts give a gross underestimate, because phytoalexins become concentrated in hypersensitive cells where they can be in considerable excess above that required for

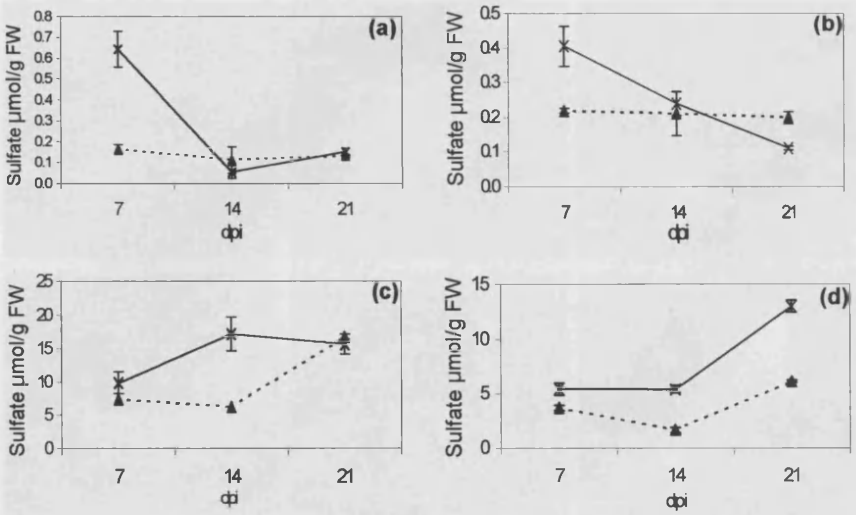

Figure 5. Temporal effects of *V. dahliae* infection on sulfate levels in resistant tomato plants. Values represent the means of three replicates with SE. Plants were inoculated with either sterile water (▲) or *V. dahliae* (x). At 7, 14, and 21 dpi, tissue samples were taken from root (a), total stem (up to node 8; data not shown), stem vascular tissue (up to node 8; b), leaf 4 (data not shown), leaf 8 (c), and leaf 15 (d) of plants and sulfate was estimated by HPLC.

pathogen inhibition (Mansfield, 2000). SEM-EDX analysis indicated that S may also be concentrated in this way.

The origin and biosynthetic pathway of elemental S formation remains unresolved. The increased sulfate levels may reflect the over-expression of sulfate transporters in response to the burden on metabolism to produce elemental S. The observed transient peaks of Cys and glutathione, particularly as these occur in the vascular tissue samples, implicate these S-containing compounds in the phenomenon. The major site of S reduction is in the leaf tissues, and glutathione would be involved in transport of this S in the vascular tissues. Reduced glutathione can also have a protective role in anti-oxidative pathogen defense reactions, and pool size can be elevated in response to these demands (Kömives et al., 1998). Notably, marked accumulation of glutathione occurred in tomato cells carrying resistance genes in response to specific fungal elicitors (May et al., 1996). Localized accumulation of glutathione and subsequent degradation together with chemical oxidative processes (Steudel et al., 1986) could result in the observed depositions. Future work will focus on the identification of Cys degradation pathways, for example the identification of a Cys desulfhydrase (Schmidt, 1987). Novel components of a pathway leading to elemental S accumulation, induced by pathogen infection, may be identified by employing gene screening procedures relying on differential expression, such as differential display. We are currently applying these approaches to this tomato model system.

MATERIALS AND METHODS

Plant Growth and Cultivation

Tomato (*Lycopersicon esculentum*) GCR 26 and GCR 218 (isogenic lines, which are susceptible and resistant, respectively, to *V. dahliae*) were either grown in Levingtons compost (fine grade 2 followed by medium grade 2) or a 1:1 sand:perlite mix in a greenhouse. Plants were fed three times a week with a full nutrient solution that contained KNO_3 (7 mM), $MgCl_2.6H_2O$ (0.9 mM), KH_2PO_4 (1.0 mM), $Mg(NO_3)_2{\cdot}6H_2O$ (1.7 mM), NaCl (0.1 mM), EDTA FeNa (0.05 mM), $Ca(NO_3)_2{\cdot}4H_2O$ (5 mM), $CaCl_2{\cdot}6H_2O$ (2 mM), $CH_3COOZn{\cdot}2H_2O$ (1.3 µM), H_3BO_3 (24.5 µM),

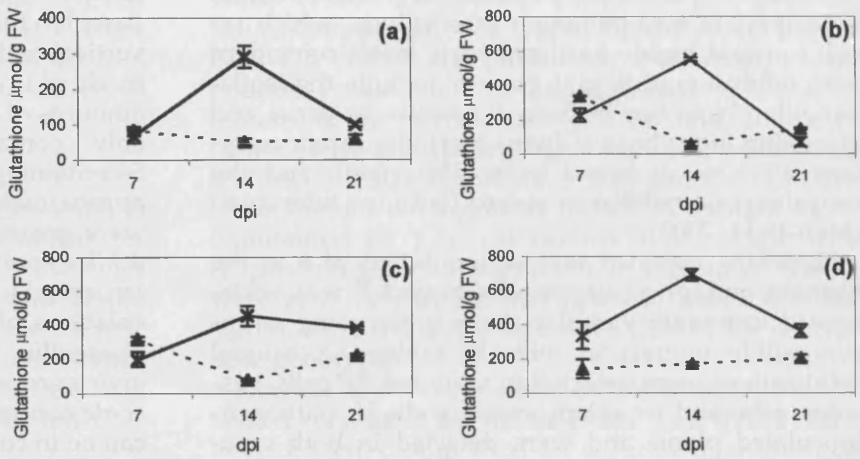

Figure 6. Temporal effect of *V. dahliae* infection on glutathione levels in resistant and susceptible tomato plants. Values represent the means of three replicates with SE. Plants were inoculated with either sterile water (▲) or *V. dahliae* (x). At 7, 14, and 21 dpi tissue samples were taken from root (data not shown), total stem tissue (data not shown) stem vascular tissue (a), leaf 4 (b), leaf 8 (c), and leaf 15 (d) of plants and glutathione content was estimated by HPLC. Glutathione levels in inoculated, susceptible plants were not significantly different from those in control plants and are omitted for clarity. A repeated experiment with stem vascular tissue produced similar data.

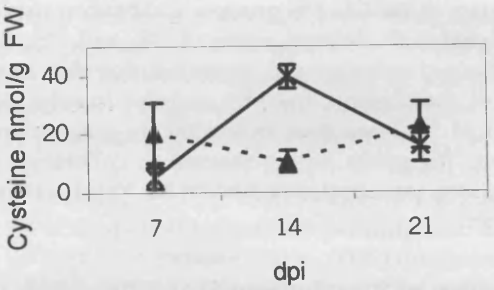

Figure 7. Temporal effects of *V. dahliae* infection on Cys in xylem tissue of resistant and susceptible tomato plants. Values represent the means of three replicates with SE. Plants were inoculated with either sterile water (▲) or *V. dahliae* (x). At 7, 14, and 21 dpi tissue samples were taken from root, total stem, stem vascular tissue, leaf 4, leaf 8, and leaf 15 of plants, and Cys was estimated by HPLC. Only stem vascular tissue (above) showed a significant change in Cys levels on pathogen infection. Cys levels in inoculated susceptible plants were not significantly different from those in control plants and are omitted for clarity. The data are representative of two comparable experiments.

$Cu(NO_3)_2 \cdot 3H_2O$ (0.6 μM), $(NH_4)_2$ Mo·$4H_2O$ (0.8 μM), FeNa EDTA (50.1 μM), $Mn(NO_3)_2 \cdot 4H20$ (9.3 μM), and 1.0 mM SO_4^{2-} supplied as $MgSO_4 \cdot 7H_2O$. The temperature was maintained at 25°C ± 3°C and supplementary illumination was supplied by Phillips 400-W high-pressure sodium lamps (Eindhoven, The Netherlands) for a 16-h daylength.

Pathogen Growth and Inoculation of Plants

V. dahliae, isolate Dvd-T5 race 1, was provided by K. Dobinson (Agriculture and Agri-food, Ontario, Canada). Spores were stored long term in 25% (v/v) glycerol at −70°C and when required were subcultured onto Czapek dox agar (Oxoid Ltd., Basingstoke, UK) and incubated at 25°C. To produce inoculum, a shake culture of *V. dahliae* was made in Czapek dox liquid medium at 25°C, 150 rpm. The resulting suspension was filtered through two layers of sterile muslin and centrifuged at 1,000*g* for 10 min. The pellet was resuspended twice in sterile milli-Q water (pH 7). Spore concentration was determined with a hemocytometer and diluted to give 1×10^7 spores mL^{-1} with milli-Q water (pH 7).

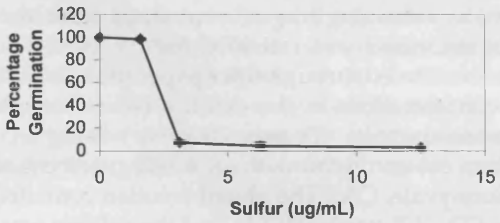

Figure 8. Inhibition of *V. dahliae* spore germination by elemental S. Values represent the percentage germination of three replicate analyses of 100 spores with SE. Probit analysis (the method commonly used to determine the potency of a toxin in a bioassay) was attempted but revealed that the data did not fit a typical dose response and therefore could not be analyzed to give an exact ED_{50} value. Therefore the ED_{50} value is expressed as between 1.56 and 3.125 μg mL^{-1}.

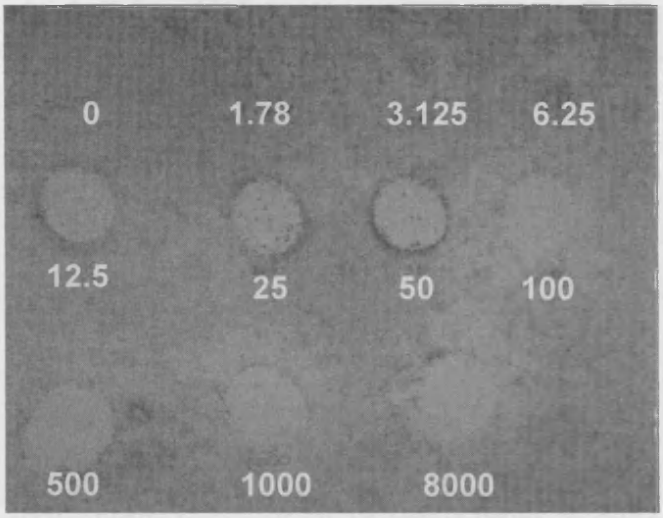

Figure 9. TLC bioassay showing toxicity of elemental S to *V. dahliae* spores and mycelium. Fifty microliters of each S solution (concentration in micrograms per milliliter is shown below each application spot) was applied to the TLC plate and a suspension of *V. dahliae* spores (1×10^6 spores mL^{-1}) sprayed on the surface. The fungus began to show as pigmented black microsclerotia at 3 d against the white background of the plate and inhibition zones were obvious at 4 d (above). The plate was analyzed for 40 d but the growth pattern did not change significantly after 4 d.

Eight-week-old plants from each cultivar were root-inoculated by pouring 50 mL per plant of the *V. dahliae* spore suspension onto the soil or sand. Control plants were sham-inoculated with 50 mL of sterile milli-Q water (pH 7). After inoculation, all plants were watered to wash the inoculum into the soil.

Quantification of *V. dahliae* from Plant Stem Tissue

Quantitative colonization analysis was performed at different stem positions (internodes 1, 8, and 15) of GCR 26 and GCR 218 soil grown, inoculated, and control plants. This was done at three time points (13, 20, and 28 d) after inoculation. Thin transverse sections of individual vascular bundles were cut with a razor blade from each of the stated internodes of three replicate inoculated and control plants and examined by light microscopy at 400×. Percentage of xylem vessels infected with fungal hyphae and percentage of vessels occluded with gels or tyloses was calculated (Cooper and Wood, 1980).

Dissection and Preparation of Xylem for Elemental S, Sulfate, and Thiol Analyses

After root-inoculation, GCR 26 and GCR 218 *V. dahliae*-inoculated and control plants were left for one of three time intervals corresponding with those used for assessment of colonization (13, 20, and 28 dpi) for preliminary quantitative analysis of elemental S. S was present in both resistant and susceptible xylem at all time points but not in controls. Therefore the experiment was repeated but using plants grown in sand culture to ensure a defined sulfate regime

and the plants were harvested at 7, 14, and 21 dpi for elemental S, sulfate, and thiol analyses. At each time point, three plants were harvested individually and the lower half of the stem up to node 8 was removed from each plant. The xylem tissue was excised by scraping away the epidermis with a scalpel, and then the vascular bundles were dissected out with forceps. All xylem tissue was frozen at −70°C and when required was comminuted in liquid nitrogen and subdivided for each analysis. For sulfate and thiol analyses roots, whole stem sections (up to node 8), leaf 4 (numbered from the base), leaf 8, and leaf 15 were additionally harvested.

Elemental S Detection by GC-MS

Dichloromethane (HPLC grade, Fisher Scientific UK. Ltd.) was added to the comminuted xylem sample (10 mL g^{-1}) and left for 10 min to extract non polar compounds. At this point a defined amount of ^{34}S standard dissolved in dichloromethane was added to quantify the ^{32}S in the sample. An attempt was made to match the level of ^{34}S added to the level of natural ^{32}S predicted from previous experiments. Extracts were then filtered through 1PS filter paper (Whatman, Clifton, NJ) into round bottom flasks and the dichloromethane removed by rotary evaporation. The remaining residue was further purified by redissolving in 20 mL hexane (HPLC grade, Fisher Scientific UK. Ltd., Loughborough, Leicestershire, UK) and running through an 8-mL silica (60A) column (Extract Clean, Alltech, Deerfield, IL). The column was further eluted with 10 mL of hexane, and the effluent containing the elemental S was collected. The hexane was removed by rotary evaporation, and the residue was resuspended in 2 mL of dichloromethane and transferred to a 5-mL glass vial from which the dichloromethane was evaporated in a stream of nitrogen gas. The final residue was then resuspended in 250 μL of dichloromethane and analyzed by GC-MS.

Elemental S was quantified (as S_8) by GC-MS using a model 5791 GC-MS (Hewlett-Packard, Palo Alto, CA), with an SGE BPX5, 25-m × 0.25-mm column and helium as carrier gas at a pressure of 80 kPa. The internal standard ($^{34}S_8$) co-elutes with $^{32}S_8$ but has a defined mass of 272 compared with 256. Single ion monitoring was used for detection and the 256 and 272 ions were analyzed in turn for 15 cycles of 50 ms to build up a peak. The column temperature regime adopted was: 35°C for 2 min, which was raised by 25°C per minute to 200°C, and then by 5°C per minute to 250°C, and finally by 15°C per minute to 320°C. The MS source temperature was 150°C and the transfer line temperature was 250°C. The ionization mode used was electron impact (+ve ion) at 70 eV. S_8 is thermally unstable at temperatures >119°C and was found to break down to S_2 during analysis. Although there was some recombination to S_8 in the cooler parts of the process, this was not complete and fragments of S_2, S_4, and S_6 were produced. Recombination of ^{32}S and ^{34}S ions also occurred giving mixed S compounds.

A cool, on-column injector with a 0.53-mm i.d. precolumn (retention gap) was used to prevent depolymerization at the start of the GC-MS process. Calibration curves were constructed with defined ratios of $^{32}S_8$ and $^{34}S_8$ to determine thermal splitting and recombination due to temperatures of the column, the MS, and the transfer line. The amount of $^{32}S_8$ was then estimated by integration of the $^{32}S_8$ and $^{34}S_8$ peaks with reference to calibration curves. Calculations were performed by an HP Pascal Chemstation computer.

Localization of S by Coupled SEM-EDX

Transverse and longitudinal sections of thickness approximately 2 mm of individual vascular bundles were excised with a razor blade (dichloromethane washed) from the first internode of *V. dahliae*-inoculated and control, susceptible and resistant plants at 12 to 16 dpi (analysis of all treatments could not be completed in 1 d and samples could not be stored) and at 28 dpi for cryofixation. A 20-nm coating of aluminum was evaporated onto tissues (gold obscured the S peak during x-ray analysis), and the samples were viewed in a JSM-6310 SEM (JEOL, Tokyo). X-ray analysis was by an AN10000 energy dispersive x-ray analyzer (Oxford Instruments Ltd, Marlow, UK).

Lyophilized samples were also prepared at 14 and 28 dpi from all treatments as cryofixed samples could not be stored. Samples were plunged into liquid nitrogen for 5 min and then transferred under liquid nitrogen to aluminum carriers for lyophilization for 12 h. Samples were then mounted on carbon adhesive discs, which were adhered to aluminum planchettes (Agar Scientific Ltd, Stansted, UK). Mounted samples were carbon coated in an E12E Vacuum Coating Unit (Edwards High Vacuum Ltd, Crawley, UK). SEM and x-ray analysis was performed as above.

Two replicate plants were used for cryofixation and a further two for lyophilization for each treatment at each time point. For analysis of relative S levels present at 12 to 16 dpi, cryofixed material was used and 25 vascular areas were randomly chosen from two randomly selected sections from each plant. Localization of S in the form of dot maps was also performed on these areas as well as in lyophilized sections at 14 dpi and with both cryofixed and lyophilized sections at 28 dpi.

Analysis of Sulfate Ions by HPLC

Sulfate was measured after the protocol of Blake-Kalff et al. (1998) by extracting 0.1 g of lyophilized plant material in 1 mL of deionized water at 90°C for 1 h, after which the extract was filtered through filter paper (no. 42, Whatman). SO_4^{2-} concentrations in the extracts were determined by ion chromatography (Dionex 2000i/sp) using an AS9SC separation column fitted with an AS9G guard column (Dionex, Sunnyvale, CA). The eluent solution consisted of 1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$, and the column was regenerated with 0.025 N H_2SO_4.

Analysis of Thiols by HPLC

Comminuted tissue (0.1 g) was extracted in 1.5 mL of 0.1 N HCl, containing 0.1 g of acid-washed polyvinylpoly-

pyrrolidone. This was mixed and left at room temperature for 1 h. Samples were then centrifuged at 10,000*g* for 5 min. Aliquots of 0.5 mL were filtered through 0.2-μm spin filters (Anachem, Luton, UK). One-hundred microliters of 0.25 M Ches (2-[*N*-cyclohexylamino] ethanesulfonic acid) was added to 100 μL of filtered sample to adjust to pH 8.0. Seventy microliters of 10 mM dithiothreitol was added before a 1-h incubation at room temperature followed by 10 μL of 25 mM monobromobimane. The components were rapidly mixed, and derivatization occurred at room temperature in the dark for 15 min. The reaction was terminated by the addition of 220 μL of 100 mM methylsulphonic acid.

Monobromobimane derivatives were then separated by HPLC using a Zorbax ODS 5-μ column (Jones Chromatography, Hergoed, UK). A gradient of 10% to 90% (v/v) methanol in 0.25% (v/v) acetic acid (pH 4.9) was used to elute the derivatives, which were detected fluorimetrically (excitation 380 nm, emission 480 nm) and compared with known standards for quantification.

Toxicity of Elemental S to *V. dahliae*

Two bioassays were devised to test toxicity of elemental S to *V. dahliae*, spore germination and mycelial growth.

Slide Bioassay of Spore Germination

Two-day-old liquid cultures of *V. dahliae* were grown in 100 mL of Czapek Dox (Oxoid) in 250-mL flasks at 150 rpm, in darkness at 25°C. Cultures were filtered through muslin and centrifuged at 3,000*g* for 10 min, and conidia were resuspended in sterile distilled water (pH 6.5) and diluted to 3×10^5 spores mL^{-1}. A 2-fold dilution series of ^{32}S (Aldrich, Milwaukee, WI) ranging from 1.6 to 100 μg mL^{-1} as well as 500, 1,000, and 8,000 μg mL^{-1} solutions were made up in dichloromethane. Fifty microliters of one of these solutions or pure solvent was pipetted into each of three 10-mm wells of a teflon-lined diagnostic slide (Merck, Darmstadt, Germany). The contents of each well was evaporated and 40 μL of the *V. dahliae* spore suspension added. Each slide was transferred to an individual Petri dish containing moistened filter paper and incubated for 15 h at 25°C. Immediately after incubation, 10 μL of 0.1% (w/v) aniline blue in lactophenol was pipetted into each well to stain conidia and arrest further growth. Conidia were considered to have germinated when germ tube length was longer than the spore diameter. Percentage germination was calculated from 100 spores from each of the three replicate wells. Data were subjected to probit analysis (Finney, 1964), the method commonly used to determine an ED_{50} or potency value from a toxicity bioassay (see Fig. 8 legend).

TLC Bioassay

A *V. dahliae* conidial suspension (1×10^6 spores mL^{-1}) was made up in Czapek Dox medium. A 20- × 20-cm TLC aluminum sheet (silica gel 60 F_{254}, Merck) was pre run in dichloromethane and allowed to dry. Fifty microliters of each of the ^{32}S solutions quoted above and pure solvent as a control was pipetted slowly onto the TLC plate and the dichloromethane allowed to evaporate resulting in approximately 25-mm diameter zones. The *V. dahliae* spore suspension was then sprayed evenly onto the plate and incubated in darkness at 25°C and 100% relative humidity. Plates were analyzed daily for 7 d and then weekly until 40 d to detect inhibition of spore germination initially and then any mycelial growth, which would eventually colonize the majority of the silica gel and could subsequently invade initial zones of inhibition. The production of pigmented, melanized microsclerotia clearly revealed fungal growth and provided a sufficiently dark background to visualize white areas where growth did not occur.

ACKNOWLEDGMENTS

The authors wish to thank Steve Croker and Paul Gaskin (IACR, Long Ashton Research Station, Bristol, UK) for their technical assistance with the GC-MS. Thanks also to Ursula J. Potter (University of Bath, Bath, UK) for her excellent assistance with the SEM-EDX.

Received August 2, 2001; returned for revision September 20, 2001; accepted October 17, 2001.

LITERATURE CITED

Bang SS, Deshpande SS, Han KN (1995) The oxidation of galena using *Thiobacillus ferrooxidans*. Hydrometallurgy **37:** 181–192

Beffa T (1993) Metabolism of elemental sulphur (S^0) during fungal spore germination. Can J Microbiol **39:** 735–741

Bell AO, Mace ME (1981) Biochemistry and physiology of resistance. *In* M Mace, A Bell, C Beckman, eds, Fungal Wilt Diseases of Plants. Academic Press, London, pp 431–486

Blake-Kalff MMA, Harrison KR, Hawkesford MJ, Zhao FJ, McGrath SP (1998) Distribution of sulfur within oilseed rape leaves in response to sulfur deficiency during vegetative growth. Plant Physiol **118:** 1337–1344

Cooper RM (1981) Pathogen-induced changes in host ultrastructure. *In* R Staples, G Toenniesen, eds, Plant Disease Control: Resistance and Susceptibility. John Wiley & Sons, Chichester, UK, pp 105–142

Cooper RM (2000) *Verticillium*-host interactions: past achievements and future molecular prospects: an overview. *In* E Tjamos, R Rowe, J Heale, D Fravel, eds, Advances in *Verticillium* Research and Disease Management. APS Press, St Paul, pp 144–150

Cooper RM, Resende MLV, Flood J, Rowan MG, Beale MH, Potter U (1996) Detection and cellular localization of elemental sulphur in disease resistant genotypes of *Theobroma cacao*. Nature **379:** 159–162

Cooper RM, Wood RKS (1980) Cell wall degrading enzymes of vascular wilt fungi, III. Possible involvement of endo-pectin lyase in *Verticillium* wilt of tomato. Physiol Plant Pathol **16:** 285–300

Cusanovich MA, Meyer TE, Bartsch RG (1991) Flavocytochrome *c*. *In* F Muller, ed, Chemistry and Biochemistry of Flavoenzymes. CRC Press, Boca Raton, FL, pp 377–399

Diwan N, Fluhr R, Eshed Y, Zamir D, Tanksley SD (1999) Mapping of *Ve* in tomato: a gene conferring resistance to the broad-spectrum pathogen *Verticillium dahliae* race 1. Theor Appl Genet **98:** 315–319

Dixon RA (2001) Natural products and plant disease resistance. Nature **411:** 843–847

Finney DJ (1964) Probit Analysis. Cambridge University Press, London, UK

Gray GO, Knaff DB (1982) The role of a cytochrome *c*-552 in the oxidation of sulfide in *Chromatium vinosum*. Biochim Biophys Acta **680:** 290–296

Hawkesford MJ, Wray JL (2000) Molecular genetics of sulphur assimilation. Adv Bot Res **33:** 159–223

Hell R (1997) Molecular physiology of plant sulfur metabolism. Planta **202:** 138–148

Ikawa M, Thomas TV Jr, Buckley LJ, Uebel JJ (1973) Sulfur and the toxicity of the red alga *Ceramium rubrum* to *Bacillus subtilis*. J Phycol **9:** 302–304

Izak RR, Stierle DB, Sims JJ (1982) Sulfur and *n*-methylformamide from the marine red alga *Erythrophyllum delesserioides*. Phytochemistry **21:** 229

Jabs T, Slusarenko AJ (2000) The hypersensitive response. *In* A Slusarenko, R Fraser, L van Loon, eds, Mechanisms of Resistance to Plant Diseases. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 279–323

Jolivet P, Bergeron E, Zimierski A, Meunier JC (1995) Metabolism of elemental sulphur and oxidation of sulphite by wheat and spinach chloroplasts. Phytochemistry **38:** 9–14

Joyard J, Fores E, Blée E, Douce R (1988) Characterization of elemental sulfur in isolated intact spinach chloroplasts. Plant Physiol **88:** 961–964

Kömives T, Gullner G, Király Z (1998) Role of glutathione and glutathione-related enzymes in response of plants to environmental stress. Ann NY Acad Sci **851:** 251–258

Kraus F, Schäfer W, Schmidt A (1984) Formation of elemental sulfur by *Chlorella fusca* during growth on L-cysteine ethylester. Plant Physiol **74:** 176–182

Kylin H, Atuma S, Hovander L, Jensen S (1994) Elemental sulphur (S_8) in higher plants: biogenic or anthropogenic origin? Experientia **50:** 80–85

Legris-Delaporte S, Ferron F, Landry J, Coster C (1987) Metabolism of elemental sulfur in wheat leaves consecutive to its foliar application. Plant Physiol **85:** 1026–1030

Leustek T, Saito K (1999) Sulfate transport and assimilation in plants. Plant Physiol **120:** 637–643

Mace ME, Bell AA, Beckman CH (1976) Histochemistry and identification of disease-induced terpenoid aldehydes in *Verticillium*-wilt-resistant and -susceptible cottons. Can J Bot **54:** 2095–2099

Mansfield JW (2000) Antimicrobial compounds and resistance: the role of phytoalexins and phytoanticipins. *In* A Slusarenko, R Fraser, L van Loon, eds, Mechanisms of Resistance to Plant Diseases. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 325–370

May MJ, Hammond-Kosack KE, Jones JDG (1996) Involvement of reactive oxygen species, glutathione metabolism, and lipid peroxidation in the *Cf*-gene-dependent defense response of tomato cotyledons induced by race-specific elicitors of *Cladosporium fulvum*. Plant Physiol **110:** 1367–1379

Moriarty DJW, Nicholas DJD (1970) Products of sulphide oxidation in extracts of *Thiobacillus concretivorus*. Biochim Biophys Acta **197:** 143–151

Pattaragulwanit K, Brune DC, Trüper HG, Dahl C (1998) Molecular genetic evidence for extracytoplasmic localization of sulfur globules in *Chromatium vinosum*. Arch Microbiol **169:** 434–444

Reinartz M, Tschäpe J, Brüser T, Trüper HG, Dahl C (1998) Sulfide oxidation in the phototrophic sulfur bacterium *Chromatium vinosum*. Arch Microbiol **170:** 59–68

Rennenberg H, Arabatzis N, Grundel I (1987) Cysteine desulphydrase activity in higher plants: evidence for the action of L- and D-cysteine specific enzymes. Phytochemistry **26:** 1583–1589

Resende MLV, Flood J, Ramsden JD, Rowan MG, Beale MH, Cooper RM (1996) Novel phytoalexins including elemental sulphur in the resistance of cocoa (*Theobroma cacao L.*) to verticillium wilt (*Verticillium dahliae* Kleb.). Physiol Mol Plant Pathol **48:** 347–359

Sasahira T, Matsui K, Arai T (1992) Oxidation of hydrogen sulfide with cell-free extract of *Hyphomicrobium neptunium* ATCC 15444. Yakugaku Zasshi **112:** 332–335

Schmidt A (1987) D-Cysteine desulfhydrase from spinach. Methods Enzymol **143:** 449–453

Schmidt A, Jäger K (1992) Open questions about sulfur metabolism in plants. Plant Mol Biol **43:** 325–349

Schmidt TM, Arieli B, Cohen Y, Padan E, Strohl WR (1987) Sulfur metabolism in *Beggiatoa alba*. J Bacteriol **169:** 5466–5472

Steudel R, Holdt G, Nagorka R (1986) On the autoxidation of aqueous sodium polysulfide. Z Naturforsch B **41:** 1519–1522

Visser JM, Robertson LA, van Verseveld HW, Gijs Kuenen J (1997) Sulfur production by obligately chemolithoautotrophic *Thiobacillus* species. Appl Environ Microbiol **63:** 2300–2305